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I. IN VIVO UTILIZATION OF ALPHA-METHYL-DL-CYSTINE BY ESCHERICHIA COLI. II. THIN-LAYER CHROMATOGRAPHY OF DANSYL AMINO ACIDS ON POLYAMIDE. III. A COMPETITIVE PROTEIN BINDING ASSAY FOR INDIVIDUAL PLASMA ESTROGENS.

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BY

JAMES CARL WESENBERG

A Dissertation

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1977

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EXTERNAL EXAMINER

ABSTRACT

PART I

IN VIVO UTILIZATION OF α-METHYL-DL-CYSTINE

BY ESCHERICHIA COLI

BY

JAMES CARL WESENBERG

A methionine-obligate (ATCC 27257) and two cysteine-obligate (ATCC 23792 and 23793) strains of E. coli were used to study the specificity in vivo of protein synthesis towards α-methyl-DL-cystine. This α-substituted amino acid analogue was not incorporated into the protein synthesized by any of these strains of E. coli.

The addition of α-methyl-DL-cystine to a basal medium containing an otherwise growth-limiting concentration of L-cysteine enhanced the total growth attained by the cysteine-obligate E. coli. By comparison to the total growth attained in media containing a growth-limiting concentration of L-cysteine plus L-methionine or α-methyl-DL-cystine, it was shown that for E. coli (ATCC 23792) that α-methyl-DL-cystine enhanced growth by serving as an alternate source of methionine. A mathematical model resulted in the development of an equation that predicted the enhanced pattern caused by this analogue. Although α-methyl-DL-cystine was also suspected to have been converted to methionine in E. coli (ATCC 23793), the results were complicated by an explained inhibitory effect on growth observed at high levels of α-methyl-DL-cystine or moderate levels of the analogue and excess L-methionine.

PART II

THIN-LAYER CHROMATOGRAPHY OF DANSYL

AMINO ACIDS ON POLYAMIDE

BY

JAMES CARL WESENBERG

The development of a two-dimensional thin-layer chromatography procedure for the separation, on polyamide, of the dansyl derivatives of the amino acids commonly found in protein is described. Of those solvent systems tested, optimal separation was achieved using water-pyridine-formic acid (93:3.5:3.5 v/v) in the first direction and benzene-acetic acid (4.5:1 v/v) in the second direction. Previous systems would require either five chromatographic solvents or four chromatographic solvents, preceded by the removal of DNS-OH from the reaction mixture, to approximate the degree of separation achieved in this two-dimensional system.

PART III

A COMPETITIVE PROTEIN BINDING ASSAY OF

INDIVIDUAL PLASMA ESTROGENS

BY

JAMES CARL WESENBERG

Preliminary work in the development of a competitive protein binding assay for plasma estrogens is described. Cytosol containing the high-affinity estrogen receptor was prepared from uterine tissue by homogenization and centrifugation at 5,000 x g, and then at 100,000 x g. Known amounts of estrone, 17-β-estradiol and estriol were added to plasma.

A small amount of the tritiated form of each estrogen was also added to monitor recovery. The plasma estrogens were extracted with ether and separated by Sephadex LH-20 column chromatography. The estrogen-containing eluates were divided for recovery determination and assay. The assay samples were incubated with the corresponding tritiated estrogen and cytosol. After incubation, dextran-coated charcoal was used to remove the free unlabelled and tritiated estrogen. The amount of tritiated estrogen bound was determined by liquid scintillation counting. Standards containing known amounts of unlabelled estrogen were assayed in the same manner. The amount of unlabelled estrogen in the assay sample was determined from the competitive protein binding curve of the standards. This value was compared to that calculated based on percent recovery. Very little assay data has as yet been generated. However, there is some evidence to suggest that the necessary developmental work has been completed.

ACKNOWLEDGEMENTS

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I wish to acknowledge my co-advisors: Dr. D.E. Schmidt, Jr., for his positive contributions, especially in regard to the mathematical model; and Dr. T.F. Draisey, for his guidance during the plasma estrogen assay study.

I would also like to acknowledge the financial support provided by the University of Windsor, the Salvation Army Grace Hospital and the National Research Council of Canada.

DEDICATION

To Cathy, my wife, for her wholehearted dedication, patience and understanding.

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ABBREVIATIONS

PART I

A	absorbance
AMC	α-methyl-DL-cystine
$AMCSO_3H$	α-methyl-cysteic acid
AMP, ATP	adenosine-5′ mono- and triphosphate
CoA	coenzyme A
$CysSO_3H$	cysteic acid
DNA	deoxyribonucleic acid
DNS, dansyl	1-dimethylaminonapthalene-5-sulfonyl
DNS-OH	1-dimethylaminonapthalene-5-sulfonic acid
fmet	N-formylmethionine
FMN, $FMNH_2$	flavin mononucleotide, oxidized and reduced form
GDP, GTP	guanine-5′ di- and triphosphate
$H_4PteGlu$	tetrahydropteroyl $(poly)_n$ glutamate
mRNA	messenger RNA
Pi	inorganic orthophosphate
PPi	inorganic pyrophosphate
Pyridoxal-P	pyridoxal-phosphate
RNA	ribonucleic acid
TLC	thin-layer chromatography
tRNA	transfer RNA

PART II

$CysSO_3H$	cysteic acid
DNS, dansyl	1-dimethylaminonapthalene-5-sulfonyl

$DNS-NH_2$	1-dimethylaminonapthalene-5-sulfonamide
DNS-OH	1-dimethylaminonapthalene-5-sulfonic acid
MetSO	methionine sulfoxide
$MetSO_2$	methionine sulfone
OH-Pro	hydroxyproline
v	volume

PART III

E_1	estrone
E_2	17β-estradiol
E_3	estriol
$^3H-E_{1,2 \text{ or } 3}$	tritiated estrone, 17β-estradiol or estriol

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PART III

PART I

IN VIVO UTILIZATION OF α-METHYL-DL-CYSTINE BY ESCHERICHIA COLI

CHAPTER I

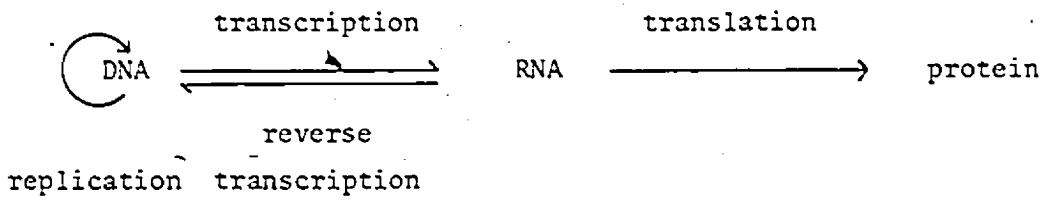
INTRODUCTION

A. SPECIFICITY IN PROTEIN BIOSYNTHESIS

DNA was first discovered in cell nuclei as long ago as 1869, by F. Miescher (1). The 1940s saw the emergence of the famous one gene - one enzyme hypothesis from the work of Beadle and Tatum (2) beginning in 1941 and the discovery by Avery and coworkers (3) that genetic information is contained in, and is transmitted by, DNA. In 1953, Watson and Crick (4, 5) postulated a double-helical structure for DNA which not only agreed with available data concerning its structural properties but also suggested a simple copying mechanism for the genetic material. Gamow (6) suggested that DNA may represent a 'code' for the incorporation of amino acids into protein. Since the work of Fischer (7) in 1906, it has been known that proteins are composed of α-amino acids linked together by peptide bonds. However, not until the early 1950s with the advent of radioisotope tracer techniques, was it proven that proteins are indeed synthesized from individual α-amino acids (8, 9).

The spark generated from the coalescence of this fundamental information produced a concentrated effort by many workers in the fields of genetics, biochemistry and molecular physics. This work has led to the development, confirmation, modification and, to date, considerable characterization of one of the central dogmas of molecular biology. It was originally thought that in living cells the flow of information is from DNA to RNA to protein, whereby three major processes are responsible for the preservation and transmission of genetic information. The first is replication, the copying of DNA to produce identical daughter molecules.

The second is transcription, the process by which the genetic message in DNA is transcribed into the form of mRNA, to be carried to the ribosomes. The third is translation, the process by which the genetic message is decoded on the ribosomes, where mRNA is used as a template in directing the specific amino acid sequence during protein biosynthesis. It was observed later that the information contained in the RNA of certain RNA viruses was able to be incorporated into the DNA of the host cell. This process known as reverse transcription was included in this central dogma, which in its modified form can be represented schematically by:



The many review articles that have appeared in the Annual Review of Biochemistry (Annual Reviews Inc., Palo Alto, CA) over the last twenty or so years have kept abreast of other review articles, books and individual developments in the field and thus probably represent one of the best 'single' sources of information.

A necessary requirement of these processes governing the flow of information in all living organisms is that the number of errors be kept at a minimum. In vivo, the frequency of introducing one naturally occurring but faulty amino acid for the correct one has been estimated by Loftfield et al. (10, 11) as being 1 in 10,000! An examination of the mechanisms responsible for the maintenance of this high degree of fidelity during translation will prove germane to the work about to be presented.

In 1954, Zamecnik and Keller (12) discovered that enzymes in the cytosol of rat liver cells are necessary for incorporation of amino acids into proteins. Several groups (13-16) showed that the reaction catalyzed by these enzymes requires ATP and is reversible. That the reaction involved the carboxyl group of the amino acid was demonstrated with hydroxylamine (14, 17). The purification of an activating enzyme specific for methionine by Berg (18), started the search for activating enzymes for the other amino acids. —Specific aminoacyl-tRNA synthetases responsible for activation of each of the twenty amino acids found in protein have been purified and characterized to varying degrees.

Thus, in amino acid activation, the first step in protein biosynthesis, ATP reacts with amino acid$_x$, with displacement of pyrophosphate, to form an enzyme-bound aminoacyl$_x$-adenylate intermediate:

$$\text{amino acid}_x + \text{ATP} + E_x \overset{Mg^{2+}}{\rightleftharpoons} \text{aminoacyl}_x - \text{AMP} \cdot E_x + PP_i$$

In this intermediate the carboxyl group of amino acid$_x$ is linked by an acid anhydride bond with the 5'-phosphate group of the AMP; the "high energy" anhydride bond activates the carboxyl group of the amino acid. The reaction is catalyzed by an enzyme (E_x, aminoacyl$_x$-tRNA synthetase) that is highly specific for amino acid$_x$.

Evidence for the existence of an 'activated' amino acid intermediate between the stage of activation and final incorporation into protein was provided by Hultin (19) and Hultin and Beskow (20). Subsequent evidence (21-23) showed that the activated amino acid is bound to an RNA species of low molecular weight (tRNA). Several workers (24-27) went on

to prove that amino acid activation and transfer to tRNA were catalyzed by the same enzyme. Various studies (26, 28, 29) presented evidence that the functional unit of tRNA for binding the aminoacyl group is a specific nucleotide end-group which consists of two cytosine nucleotides followed by a terminal adenine nucleotide on the 3′ end of the tRNA. Holley et al. (30) were the first to provide the complete structure of a tRNA molecule, i.e., yeast $tRNA^{Ala}$. Several other tRNAs have since been characterized. There is more than one specific tRNA for each amino acid (31, 32). They are referred to as isoaccepting species of tRNA. In some cases the only differences are in the primary structures of the tRNAs, e.g., two of the three $tRNA^{Tyr}$s of E. coli and two of the five $tRNA^{Ser}$s from yeast, but in some there are base substitutions in the anticodons which are necessary to recognize synonym codons in the mRNA, e.g., the two $tRNA^{Ser}$s and the two $tRNA^{Leu}$s of E. coli. The reason for isoaccepting species differing only in primary structure is not understood in procaryotes. However, in eucaryotes different populations of tRNAs and even synthetases have been found in different cell compartments, i.e, cytosol, mitochondria and chloroplasts (33-36).

Therefore, during the formation of aminoacyl-tRNA, the second step in protein biosynthesis, the $aminoacyl_x$ moiety is transferred from the AMP to the 3′ end of a corresponding specific tRNA molecule ($tRNA^x$), followed by the release of $aminoacyl_x$-tRNA synthetase, AMP and $aminoacyl_x$-$tRNA^x$:

$$\text{aminoacyl}_x\text{-AMP}\cdot E_x + \text{tRNA} \overset{Mg^{2+}}{\rightleftharpoons} \text{aminoacyl}_x\text{-tRNA}^x + \text{AMP} + E_x$$

The aminoacyl group is esterified by its activated carboxyl to the free 2′ - hydroxyl group of the terminal AMP residue at the 3′ end of $tRNA^x$ which, like all tRNAs, bears the 3′ terminal sequence CpCpA-OH. An equilibrium mixture of the aminoacyl esters subsequently forms. This reaction is also catalyzed by $aminoacyl_x$-tRNA synthetase. The tRNA to which the amino acid is ultimately linked is governed by the mutual recognition of $aminoacyl_x$-tRNA synthetase and a species of $tRNA^x$, the anti-codon of which is complementary to one of the one or more mRNA codons that specify $amino\ acid_x$.

In 1958, Crick (37) first proposed the adaptor hypothesis which states that adaptor molecules (now tRNA) intervene between the amino acid and the polynucleotide (mRNA) so as to simultaneously bind and interact with, selectively, specific polynucleotide coding units to one portion of the adaptor molecule and an appropriate amino acid to another. In 1959, Preiss et al. (26) found evidence for the existence of tRNA molecules 'specific' for single amino acids. Chapeville et al. (38, 39) in 1962 and 1963, validated Crick's concept. Cysteinyl-$tRNA^{Cys}$ was formed by means of the cysteinyl-tRNA synthetase / $tRNA^{Cys}$ preparation from E. coli, and then chemically modified to alanyl-$tRNA^{Cys}$. When alanyl-$tRNA^{Cys}$ was introduced to protein-synthesizing systems, alanine was incorporated into protein in place of cysteine. Thus the tRNA does serve as an adaptor to which the amino acid binds so that it can be adapted to the nucleotide triplet language of the genetic code. The incorporation of a natural amino acid, whether right or wrong with respect to the tRNA to which it is linked, appeared to depend only on

the mutual recognition of the tRNA moiety and the mRNA. Stulberg and Isham (40) showed that there was no direct specificity link between the amino being activated and the tRNA to which the amino acid would be ultimately bound. The site of amino acid attachment to the tRNA (the AMP on the CpCpA-OH terminus) conferred no specificity to the amino acid being activated but does have a role in the binding to the aminoacyl-tRNA synthetase. In vitro studies (41-45) show that isoleucyl-tRNA synthetase can activate isoleucine and valine in the absence of $tRNA^{Ile}$. Addition of $tRNA^{Ile}$ then results in the enhancement of specificity in favour of isoleucine, rapid hydrolysis of the activated valine complexes and exclusive formation of isoleucyl-$tRNA^{Ile}$. The addition of the appropriate tRNA performs an error-correcting function, perhaps by affecting enzyme conformation. It is impossible to determine whether similar effects occur in vivo.

In summary, the first two steps of protein synthesis are quite interdependent. Despite whatever mechanisms to maintain fidelity exist in the remaining steps of protein biosynthesis, the specificity of the recognition and binding sites of each aminoacyl-tRNA synthetase for the proper amino acid and tRNA and those of each tRNA for the proper aminoacyl-tRNA synthetase and the complementary mRNA is crucial to reducing errors during translation.

After the formation of the aminoacyl-tRNAs, the amino acids are ready for incorporation into a polypeptide chain. Glutamine is one of the two exceptions to this statement. Glutaminyl-tRNA synthetase activates glutamic acid and transfers it to a tRNA, the anticodon of which is comple-

mentary to the mRNA codon that specifies glutamine. Thus, glutamyl-$tRNA^{Gln}$ must be converted to glutaminyl-$tRNA^{Gln}$. In the presence of ATP, Mg^{2+} and glutamine, an aminotransferase catalyzes the required transamination reaction (46).

In procaryote systems two tRNAs specific for methionine are present. The methionine which becomes bound to $tRNA_f^{Met}$ is formylated enzymatically to produce N-formylmethionyl-$tRNA_f^{Met}$. This particular aminoacyl-tRNA species is the only one recognized by those components involved during the initiation stage of protein biosynthesis (47-50). Two methionine specific tRNAs are also present in eucaryote systems. Although neither methionyl-tRNA complex is formylated, only one of these (met-$tRNA_{f*}^{Met}$) is able to promote initiation (51, 52). In both procaryote and eucaryote systems the other species met-$tRNA^{Met}$ are only utilized for the incorporation of methionine in the interior of a polypeptide chain.

The actual biosynthesis of a polypeptide chain is accomplished by two multi-step processes referred to as initiation and elongation. Subsequent release of the completed polypeptide takes place in a multi-step process called termination. Two excellent reviews by Lucas-Lenard and Lipmann (53) and Haselkorn and Rothman-Denes (54) served as the source of information for the following brief descriptions of these processes.

Initiation in procaryotes involves the following steps. A protein initiation factor IF-3 binds to the 30-S ribosomal subunit. Another protein initiation factor IF-2 joins the complex. Specific

recognition of the mRNA (probably recognition of the initiator codon AUG or GUG) by IF-3 and reaction enhancement by IF-2, a 30-S·IF-3·IF-2·mRNA complex is formed. IF-2 directs the specific attachment of fmet-$tRNA_f^{Met}$ to the mRNA, i.e. hydrogen bonding of the anticodon (CAU) of the fmet-$tRNA_f^{Met}$ to the complementary mRNA initiator codon (AUG or GUG). GTP is also bound to the complex. A third protein initiation factor IF-1 reportedly acts in conjunction with IF-2 in the attachment of fmet-$tRNA_f^{Met}$ to the mRNA. After release of IF-3 the 'initiation complex' (30-S·IF-2·GTP·fmet-$tRNA_f^{Met}$·mRNA·IF-1) accepts the 50-S ribosomal subunit. IF-1 enhances the IF-2-mediated hydrolysis of GTP. IF-1, IF-2, GDP and P_i are subsequently released. The functional ribosome 70S·fmet-$tRNA_f^{Met}$·mRNA has two important sites, the A (acceptor) site and the P (peptidyl) site.

At this point the fmet-$tRNA_f^{Met}$ initiator mRNA codon occupies the P site and the second mRNA codon occupies the A site.

Initiation in eucaryotes occurs in an analogous fashion. That non-formylated methionyl-$tRNA_f^{Met}$ is used in initiation has been described. Most eucaryote methionyl-$tRNA_{f*}^{Met}$ complexes (but not methionyl-$tRNA^{Met}$) can accept a formyl group from a bacterial donor. The resultant fmet-$tRNA_{f*}^{Met}$ can be used for initiation in both procaryote (55) and eucaryote (56) systems in vivo. Initiation of protein biosynthesis in mitochondria and chloroplasts resembles that of procaryotes since fmet-tRNA is utilized.

The amino acid specified by the second mRNA codon is incorporated by the process of elongation which is similar in both procaryotes and eucaryotes. First, a protein elongation factor EF-T, composed of two

subunits EF-T_u and EF-T_s, combines with GTP to form an EF-T_u·GTP complex, with release of EF-T_s. The EF-T_u·GTP complex binds an aminoacyl-tRNA. Neither fmet-$tRNA_f^{Met}$ or met-$tRNA_{f*}^{Met}$ can be bound. Mutual recognition between the anticodon of the tRNA moiety and the mRNA codon, results in this complex binding to the ribosome in such a way that the aminoacyl-tRNA is positioned at the A site, with the anticodon hydrogen bonded to the complementary mRNA codon. Simultaneously, the bound GTP is hydrolyzed to GDP and P_i and the EF-T_u·GDP complex and the P_i are released. Then the aminoacyl-tRNA attacks through its nitrogen atom the terminal carboxyl of the neighbouring initiator (fmet-$tRNA_f^{Met}$ or met-$tRNA_{f*}^{Met}$) or peptidyl-tRNA resulting in the formation of a peptide bond. This reaction is catalyzed by peptidyl transferase present in the 50-S ribosomal subunit. In translocation, the third step of elongation, the ribosome moves to the next codon on the mRNA and simultaneously shifts the peptidyl-tRNA from the A site to the P site. The tRNA which donated the peptidyl moiety is released during the process. This step requires a protein elongation factor EF-G and the hydrolysis of two molecules of GTP. The elongation cycle can now be repeated.

When translocation places one of the so-called nonsense codons (UAA, UGA or UAG) in the A site, no aminoacyl-tRNA is bound and termination occurs. One of two proteins, R, on recognition of UAA or UAG or R_2 on recognition of UAA or UGA, binds to the ribosome. The ester bond between the polypeptide chain and the last tRNA is hydrolyzed, apparently by peptidyl transferase, whose specificity and catalytic action is modified by the R factor. The polypeptide, the last tRNA and the mRNA leave the

ribosome which dissociates to the 30-S and 50-S subunits. A third protein factor, S, enhances the rate of termination presumably by lowering the K_m for binding of terminal trinucleotides to the ribosome. A similar process occurs in eucaryotes: only one 'R' protein has been found; no 'S' equivalent is known, and GTP is required.

The degree of fidelity which has been attained during amino acid activation and formation of aminoacyl-tRNAs can not be improved upon, but only maintained during the later stages of protein biosynthesis. Specificity during initiation and especially elongation, relies on the mutual recognition of tRNA anticodons and the complementary mRNA codons. The process of initiation is obligatory in order to establish the proper frame of reference for translation of the mRNA, i.e., recognition of a site that specifies: "start translation with this codon and continue by translating three nucleotides (one codon) at a time." However, special specificity requirements are necessary for this. The first is that only fmet-$tRNA_f^{Met}$ or met-$tRNA_{f*}^{Met}$ be utilized during initiation through recognition of the mRNA initiation codons AUG or GUG, i.e., prevent utilization of met-$tRNA^{Met}$ the anticodon of which is complementary to AUG and val-$tRNA^{Val}$ the anticodon of which is complementary to GUG. In procaryotes the protein initiation factor IF-2 appears to provide the required specificity in that this factor directs the attachment of only fmet-$tRNA_f^{Met}$ to the mRNA-initiator codon on the 30S ribosomal subunit. All other aminoacyl-tRNAs including met-$tRNA_f^{Met}$, are inactive (57), which suggests the need for the blocked amino acid group. However, blocking of the amino group is not sufficient, since most aminoacyl-tRNAs with a masked

amino group are not recognized (58), but there are exceptions, e.g., N-acetylphenylalanyl-tRNA^{Phe} (59, 60) and N-acetylvalyl-tRNA^{Val} (60, 61). The eucaryote system appears to accept in vitro either met-tRNA$_{f*}^{Met}$ or fmet-tRNA$_{f*}^{Met}$ (56), i.e., the free or blocked amino group, which suggests that perhaps the conformation of the tRNA moiety is important. Similarly, although acceptance of met-tRNA^{Met} would not alter the amino acid in the first position, this species and all other aminoacyl-tRNAs are unacceptable. During elongation, the converse is true. It is vital that fmet-tRNA$_f^{Met}$ is not incorporated in response to an internal AUG or GUG mRNA codon, since the blocked amino group would prevent peptide bond formation and further translation. The EF-T_u GTP complex fulfills this obligation by refusal to bind fmet-tRNA$_f^{Met}$ (62). Furthermore, uncharged tRNAs (63, 64), N-acetylated aminoacyl-tRNAs (64) or nitrous acid-deaminated aminoacyl-tRNAs (65) are also not bound by this complex. This suggests the requirement for a free amino group. The conformation of the tRNA structure may also be involved in the selection process since met-tRNA$_f^{Met}$ is not bound (62).

In summary: the initiation process recognizes the starting point and thereby establishes the frame of reference for proper translation of the mRNA; the specificity of elongation results in the rejection of initiator aminoacyl-tRNAs and aminoacyl-tRNAs in which the amino group of the aminoacyl moiety has been removed or altered; and the specificity associated with amino acid activation and formation of aminoacyl-tRNAs ensures that each amino acid is linked only to its corresponding tRNA. These factors ensure that a naturally occurring but faulty amino acid is

rarely, if ever, incorporated in place of the correct one.

The use of several aminoacyl-tRNA analogues, i.e., those in which the amino group of the aminoacyl moiety has been removed or chemically altered, for the study of the specificity of initiation and elongation has been discussed. The specificity of amino acid activation and formation of aminoacyl-tRNAs and of translation in general has been investigated with a variety of amino acid analogues. Sharon and Lipmann (66) have found that several tryptophan analogues stimulate hydroxamic acid formation with purified pancreatic tryptophan-activating enzyme. p-Fluorophenylalanine, ethionine, norleucine and selenomethionine are activated (67, 68). Selenomethionine (69) and p-fluorophenylalanine (70) are even incorporated into protein in place of methionine and phenylalanine, respectively. Neale (71) showed that seleno-DL-methionine, α-methyl-DL-methionine, L-ethionine and seleno-DL-ethionine were activated by methionyl-tRNA synthetase and that all of these, except α-methyl-DL-methionine, could be incorporated in place of methionine or leucine, which suggests that this analogue is activated by both methionyl- and leucyl-tRNA synthetases (71). Norleucine can be attached to either $tRNA_f^{Met}$ or $tRNA^{Met}$ and norleucyl-$tRNA_f^{Met}$ can be formylated by the appropriate E. coli enzyme (72). Amino acid phosphonate derivatives, i.e., amino acid analogues lacking a carboxyl group, are not activated and can result in the inhibition of activation (71, 73). In studies on the specificity of valyl-tRNA synthetase from E. coli, Owens and Bell (74) used valine analogues (L-norvaline, L-α-aminobutyrate, cycloleucine, isovaline, and α-aminoisobutyrate and their corresponding amines) to show

that the presence of an α-carboxyl group in the molecule was not essential for activation. One research group (75-77) reported the activation and incorporation of two S-substituted analogues, S-(p-chlorophenyl)-L-cysteine and S-(1,2,3,4-tetrahydro-2-hydroxy-1-napthyl)-L-cysteine, but cysteinyl-tRNA synthetase was found not to be responsible for activation.

Those analogues which are incorporated into protein have been used as useful probes in the determination of the role that the corresponding natural amino acid plays in determining the conformation, and thus the enzymic and immunological properties of specific proteins. Schlesinger (78) studied the effect of 7-azatryptophan and tryptazan (α-amino-β-3-(indazole) propionic acid) as amino acid analogues of tryptophan on alkaline phosphatase activity and on protein synthesis activity in E. coli. Sykes et al. (79) studied m-fluorotyrosine-containing alkaline phosphatase.

The effects of amino acid analogues on cellular metabolism, other than protein biosynthesis have been studied in both in vitro and in vivo systems. The effect of many analogues on the growth of microorganisms has been reviewed by Richmond (80). α-Methyl-tryptophan, although not incorporated into protein, has been found to cause a stimulation of the activity of tryptophan pyrolase which results in the stimulation of hepatic gluconeogenesis and of amino acid metabolism (81).

B. METHIONINE BIOSYNTHESIS IN E. COLI

Methionine biosynthesis in microorganisms and plants has recently been comprehensively reviewed by Flavin (82). The biosynthesis of methionine in bacterial systems has been studied most often in E. coli

and/or Salmonella typhimurium. In both bacterial, methionine is biosynthesized from homoserine in four sequential reactions:

L-homoserine + succinyl-CoA ⟶ O-succinyl-L-homoserine + CoA (1)

O-succinyl-L-homoserine + L-cysteine ⟶ L-cystathionine + succinate (2)

L-cystathionine + H_2O ⟶ L-homocysteine + pyruvate + NH_3 (3)

L-homocysteine + N^5-CH_3-H_4PteGlu$_n$ ⟶ L-methionine + H_4PteGlu$_n$ (4)

In 1959, Davis and coworkers (83) reported that certain succinate-requiring mutants of E. coli grew in the absence of succinate when provided with a mixture of methionine + lysine + threonine. This information prompted Rowbury and Woods to determine whether succinate is required for the formation of an intermediate in the bacterial metabolic pathway to methionine. In a series of reports these workers confirmed reactions 1 and 2 in E. coli (84-89) and S. typhimurium (88). Reactions 1 and 2 are catalyzed by homoserine O-transuccinylase and cystathionine γ-synthase, respectively. Flavin and associates in studies with S. typhimurium (90-92) and E. coli (92) found that cystathionine γ-synthetase requires pyridoxal-P; and furthermore, in the absence of cysteine, this same enzyme catalyzes the decomposition of O-succinylhomoserine:

O-succinyl-L-homoserine + H_2O ⟶ α-ketobutyrate + NH_3 + succinate (5).

Rowbury and Woods (85, 88, 89, 93-96) reported that the synthesis of these two enzymes in both bacteria is repressed by methionine and that homoserine O-transuccinylase is subject to feedback inhibition by methionine. Lee et al. (97) found that enzyme inhibition also occurs in the presence of S-adenosylmethionine, but is greatest in the presence of both these compounds. The mechanism of the reaction for the formation of

O-succinylhomoserine has not been studied in detail, but Flavin (82) suggested the intermediary formation of a succinylated enzyme. Since cystathionine γ-synthase is the only pyridoxal-P enzyme catalyzing elimination and replacement of substituents on C-4 of an amino acid, the mechanism of reactions 2 and 5 have been investigated by Flavin and co-workers (98-104).

In 1953 and thereafter, Wisejundera and Woods (105, 106) found that many strains of E. coli contain an enzyme, ß-cystathionase, which catalyzes the formation of homocysteine from cystathionine. Flavin and associates (90, 92) showed the presence of this enzyme in S. typhimurium as well as E. coli. This enzyme also requires pyridoxal-P (92, 106). Woods et al. (93, 96, 107-110) reported that the synthesis of ß-cystathionase is repressed by methionine.

In E. coli the transmethylation of homocysteine to give methionine can proceed in two ways. In both reactions the source of the methyl group is an N^5-CH_3-H_4PteGlu$_n$ derivative synthesized specifically for homocysteine transmethylation by reduction of N^5-CH_2=H_4PteGlu$_n$ (82). The latter can arise in two ways: H_4PteGlu$_n$ can be formylated to give N^5-CH=H_4PteGlu$_n$ and subsequently reduced; or it can arise directly from the reaction of H_4PteGlu$_n$ and serine. The formation of N^5-CH_3-H_4PteGlu$_n$ from N^5-CH_2=H_4PteGlu$_n$ is catalyzed by methylene tetrahydrofolate reductase (82).

Homocysteine can be transmethylated by either of two enzymes: non-vitamin B_{12}-dependent homocysteine transmethylase, which transfers the methyl group from polyglutamate forms of N^5-CH_3-H_4PteGlu$_n$ (111); or

vitamin B_{12}-dependent homocysteine transmethylase which transfers the methyl group from N^5-CH_3-H_4PteGlu, to homocysteine in the presence of a coenzyme form of vitamin B_{12}. The latter is more efficient; the turnover numbers of the two E. coli enzymes are 14 and 800, respectively (112). The former suffices in the absence of vitamin B_{12}.

The characterization of the vitamin -B_{12} dependent reaction was made possible through isolation of an E. coli mutant that lacked the non-vitamin B_{12}-dependent enzyme (113). Woods and coworkers showed that cyancobalamin stimulates methionine synthesis (114) and specifically that cyanocobalamin increases methionine synthesis from homocysteine and serine (115). Further work showed that either serine or formate, H_4PteGlu$_1$ and cobalamin are required (116). Hatch et al. (117) reported the need for a flavin-reducing system, serine hydroxymethylase, a B_{12}-containing enzyme and some other unidentified protein component. In 1962, it was reported by Woods' laboratory (118) that synthetic free methylcobalamin could serve in place of N^5-CH_3-H_4PteGlu$_1$ as a substrate for homocysteine transmethylation. This observation has since been confirmed (119). The overall reaction appears to involve the transfer, from nitrogen to sulfur, of a methyl group without its bonding electrons with the intermediary formation of a methyl-cobalt bond to the enzyme-bound cobalamin (82, 120). In vitro, a reducing system and catalytic amounts of S-adenosylmethionine (121) are necessary. The rate of catalysis is highest with $FMNH_2$ + dithiothreitol (120). It is presumed that a reducing system is required to maintain the monovalent cobalt atom in order that it can abstract the methyl group from N^5-CH_3-H_4PteGlu$_1$ (120).

The catalytic amounts of S-adenosylmethionine are thought to be required to shift the equilibrium between cob(I)alamin and cob(II)alamin. The reducing system used *in vivo* is not known. Nor is it known whether S-adenosylmethionine is required *in vivo* (119).

Woods *et al.* (93, 96, 107-110) reported that the enzymes required for homocysteine transmethylation are repressed by methionine. Katzen and Buchanan (122) observed methylene tetrahydrofolate reductase synthesis was partially repressed by methionine or vitamin B_{12}. The same is true for non-vitamin B_{12}-dependent homocysteine transmethylase (123, 124).

C. UTILIZATION OF α-METHYL-DL-CYSTINE

In this laboratory there has been considerable activity in the synthetic preparation (125-130), characterization (131, 132) and elucidation of biological activities of α-substituted-DL-cystines (129, 130, 133) and β-*p*-substituted-phenylcystine derivatives (134).

A number of reasons prompted this present research undertaking. Previous studies (75-77) have shown that certain S-substituted analogues are activated and incorporated into protein, although activation is not mediated by the highly specific L-cysteinyl-tRNA synthetase. The primary objective of this work was to investigate the specificity of the translation process of protein biosynthesis towards the α-methyl-DL-cystine. Schneider (130) has done preliminary studies with [^{3}H]-AMC. The results of these experiments using classical *in vitro* techniques with cell-free protein synthesizing systems from bovine liver and *E. coli B* did not show conclusively whether AMC was or was not incorporated into protein. The radioactivity of the protein synthesized was just slightly greater

than that of the background activity in the control samples. The only way to determine whether incorporation had occurred was to analyze the protein in an attempt to determine α-methyl-cysteine by means other than radioactivity. However, since very little protein is synthesized in these *in vitro* systems, this was not possible.

The work presented here reports the results of experiments designed to determine whether AMC is incorporated into the protein synthesized *in vivo* by methionine - or cysteine - obligate strains of *E. coli*. *In vivo* protein synthesis provides a sufficient amount of protein to determine the specificity of protein synthesis by confirming the presence or absence of the analogue using chemical methods of analysis. Also, the use of non-tritiated AMC safeguards against the detection of a false positive for incorporation. *In vivo*, some of the AMC may be metabolized and eventually used in the synthesis of other amino acids which could be incorporated into protein.

Substitution of amino acid analogues could lead to alteration of the conformation, and thus the enzymic and immunological properties, of specific proteins. The key role of L-cysteine in determining the conformation of polypeptides (disulfide bridges) and the necessity of a free -SH group for the biological activity of some enzymes, make this amino acid and its analogues a very interesting area for study. The above-mentioned study can therefore be described as the continuation of the research for a 'cysteine' probe which would be used to evaluate the role of cysteine in specific proteins. In fact, the methionine-obligate strain of *E. coli* was chosen because it is constitutive for alkaline phosphatase. If the

analogue was found to be incorporated into protein, the abundant AMC-containing alkaline phosphatase could be readily purified for investigation of the role of cysteine in this protein.

The utilization of AMC, other than direct incorporation into protein, and the effect this has on the metabolism of cysteine in cysteine-obligate strains of E. coli has also been investigated.

CHAPTER II

EXPERIMENTAL

A. MATERIALS

Nutrient agar was obtained from Difco Laboratories (Detroit, MI 48201); dansyl-Cl from Pierce Chemical Co. (Rockford IL 61105); and polyamide sheets (Macherey-Nagel Polygram Polyamide -6) from Brinkman Instruments (Toronto M9W 4Y5).

α-Methyl-DL-cystine was prepared as outlined in detail elsewhere (125).

All other chemicals were reagent grade.

B. METHODS

1. Bacterial Strains and Growth Conditions

The Escherichia coli strains used in this study were obtained from the American Type Culture Collection (Rockville, MD 20852). They included a methionine-obligate strain, ATCC 27257 and two cysteine-obligate strains, ATCC 23792 and ATCC 23793. All strains were maintained by periodic subculturing on nutrient agar.

E. coli ATCC 27257 was grown in a basal medium (Medium 1) consisting of: 0.12 M Tris buffer (pH 7.4); 4×10^{-4} M $MgSO_4$; 10^{-2} M NaCl; 2×10^{-5} M KCl; 10^{-2} M NH_4Cl; 3×10^{-4} M KH_2PO_4; 5×10^{-6} M $ZnSO_4$; and 0.3% glucose. Glucose was sterilized separately and added at the time of innoculation. E. coli ATCC 23792 and 23793 were grown on the same medium with the addition of 3×10^{-8} M thiamine (Medium 2). Depending on the strain and the nature of each experiment, the appropriate amino acid(s) was also added. In order to standardize the number of cells with which

each culture was innoculated, a cell suspension in media base with an A at 620 nm of 0.020 was prepared from the stock culture, of which 1.0 ml was used as the innoculum. Cells were routinely grown in 100-ml cultures in 300-ml Erlenmeyer flasks on a rotary shaker at 30°C. Growth was monitored by measuring the A of a culture aliquot at 620 nm.

2. Amino Acid Uptake

For some cultures, the disappearance of the amino acid(s) from the medium was monitored throughout the growth cycle. After determination of A, the culture aliquot was centrifuged at 15000 x g for 10 min. A 1.0-ml aliquot of the culture supernatant was acidified with 0.05 ml of concentrated HCl. The amino acid composition of a 0.2-ml aliquot of the acidified culture supernatant was determined using a Beckman Model 120C amino acid analyzer. The initial amino acid composition was determined using an acidified aliquot of media taken before innoculation. The concentration of each amino acid in the culture supernatant is reported as the area under the corresponding peak observed on the chromatogram.

3. Protein Analysis

To determine whether either strain incorporated α-methylcysteine directly into protein, the following procedure was used. When the culture reached the stationary phase the cells were harvested by centrifugation at 15000 x g for 20 min; washed twice with about 200 ml and suspended in about 25 ml of 0.01 M Tris buffer (pH 7.5). The cells were lysed using a sonic dismembrator (Model 300, Artek Systems Corp.) and the cellular debris removed by centrifugation at 15000 x g for

20 min at 4°C. An equal volume of cold 10% trichloroacetic acid was added to the cell-free extract. The precipitated protein was collected by centrifugation at 15000 x g for 15 min at 4°C and washed with 100 ml each of warm 95% ethanol, warm 95% ethanol-ether-chloroform (2:2:1 v/v) and warm ether. This protein was dissolved in 10 ml of 0.1 N NaOH, dialyzed for 8 h against three changes of 4000 ml each of deionized distilled water and then lyophilized.

A 5-mg sample of the protein was subjected to performic acid oxidation according to the method of Moore (135), and then hydrolyzed in 6N HCl at reduced pressure at 100° C for 18h. The hydrolyzate was evaporated to dryness, dissolved in 1.0 ml of 0.5 M $NaHCO_3$, and transferred to a small test tube. If necessary, the pH of this solution was adjusted upward to pH 9 with the addition of solid $NaHCO_3$. Then 9 mg of dansyl-Cl in 1 ml of acetone was added to the test tube, which was then sealed with parafilm and kept in a 37°C water bath for 60 min. Then, after the addition of 0.5 ml of 88% formic acid, the dansyl-amino acid mixture was extracted exhaustively with a total of about 25 ml of ether.

The dansyl-amino acids remaining in the aqueous layer were spotted in a corner about 1.5 cm from each edge of a 10 x 10 cm polyamide sheet. The chromatogram was developed in the first dimension in water-pyridine-acetic acid-formic acid (93:4.5:0.8:1.0 v/v), dried in a stream of cold air, and developed in the second dimension in water-formic acid (100:3 v/v). The separated dansyl-amino acids were visualized by long-wave ultraviolet radiation. The R_f values for those dansyl-amino acids which, if present, remain in aqueous phase (α - and ε-DNS -lysine,

DNS-histidine, DNS-arginine, DNS-cysteic acid, and DNS-α-methyl-cysteic acid) were determined using standards prepared and chromatographed as outlined above. DNS-OH, a side product of the dansylation reaction, also remains in the aqueous phase.

In some cases at the end of the growth phase, the trichloroacetic acid-precipitable protein was isolated, dialyzed, oxidized with performic acid and hydrolyzed as described above. The ratio of cysteic acid to methionine sulfone in the hydrolyzate was determined using a Beckman Model 120C amino acid analyzer.

CHAPTER III

RESULTS AND DISCUSSION

A. NON-INCORPORATION OF AMC INTO PROTEIN

A series of cultures of the methionine-obligate E. coli ATCC 27257 were grown in Medium 1 containing L-methionine (133 μM) and varying concentrations of AMC (0-1850 μM). The experiment was repeated several times and Figure 1 shows typical growth curves and amino acid uptake results for three different amino acid compositions. At concentrations of AMC approximately equal or lower to that of the methionine, the growth curves were similar to those cultures with only methionine present. At concentrations of AMC greater than methionine, the lag phase decreased as the concentration of AMC increased. The amino acid uptake results showed that methionine was completely taken up by the cells in all cultures. For those cultures which contained AMC, regardless of concentration, only 10-15% of the AMC disappeared from the medium.

Figure 2 shows a typical chromatogram of dansyl-amino acid standards which, if present, remain in the aqueous phase after ether extraction. The protein from cells grown in media containing methionine and AMC (0, 185, 1850 μM) was isolated and analyzed as described. DNS-cysteic acid was detected on all chromatograms. However, no DNS-α-methyl-cysteic acid was detected on chromatograms corresponding to the protein isolated from those cells grown in the presence of AMC.

The growth curves for the cysteine-obligate E. coli ATCC 23792 grown in Medium 2 containing increasing concentrations of L-cysteine (14-560 μM) are shown in Figure 3. The final amount of growth (the

FIGURE I

GROWTH AND AMINO ACID UPTAKE OF E. COLI ATCC 27257

Legend

Three cultures (A, B and C) of E. coli ATCC 27257 were grown in Medium 1 containing L-methionine (133 µM). In addition, the media of B and C contained AMC, 185 µM and 1850 µM, respectively. Dashed lines, labelled A, B and C represent growth (A) against time and solid lines A, B and C show the disappearance of L-methionine from the media (peak area). The solid lines B´ and C´ represent the disappearance of AMC.

FIGURE I

GROWTH AND AMINO ACID UPTAKE OF E. COLI ATCC 27257

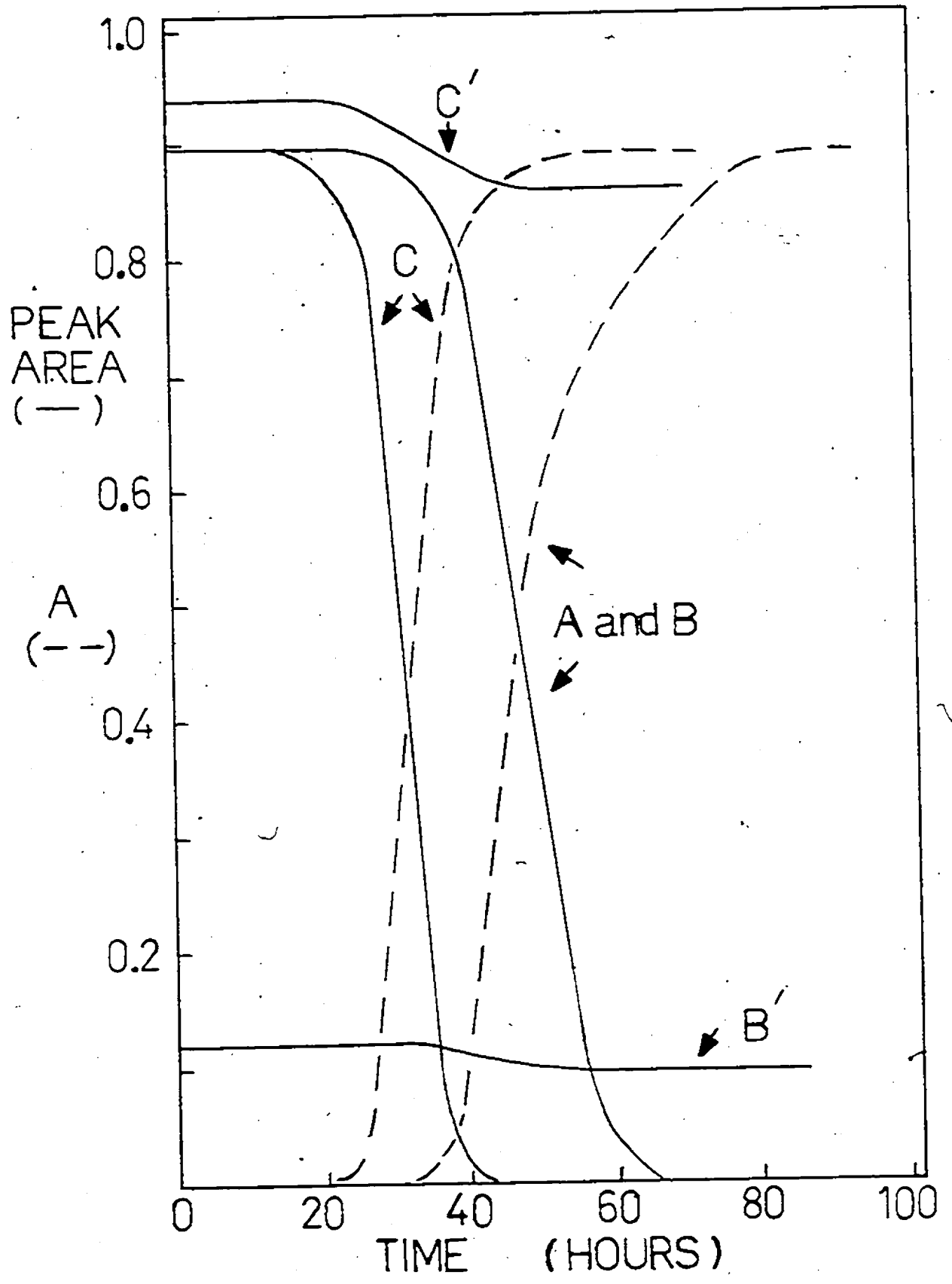


FIGURE 2

TLC OF DANSYL-AMINO ACID STANDARDS

Legend

Prepared and chromatographed as described in CHAPTER II, EXPERIMENTAL, B, 3.

FIGURE 2

TLC OF DANSYL-AMINO ACID STANDARDS

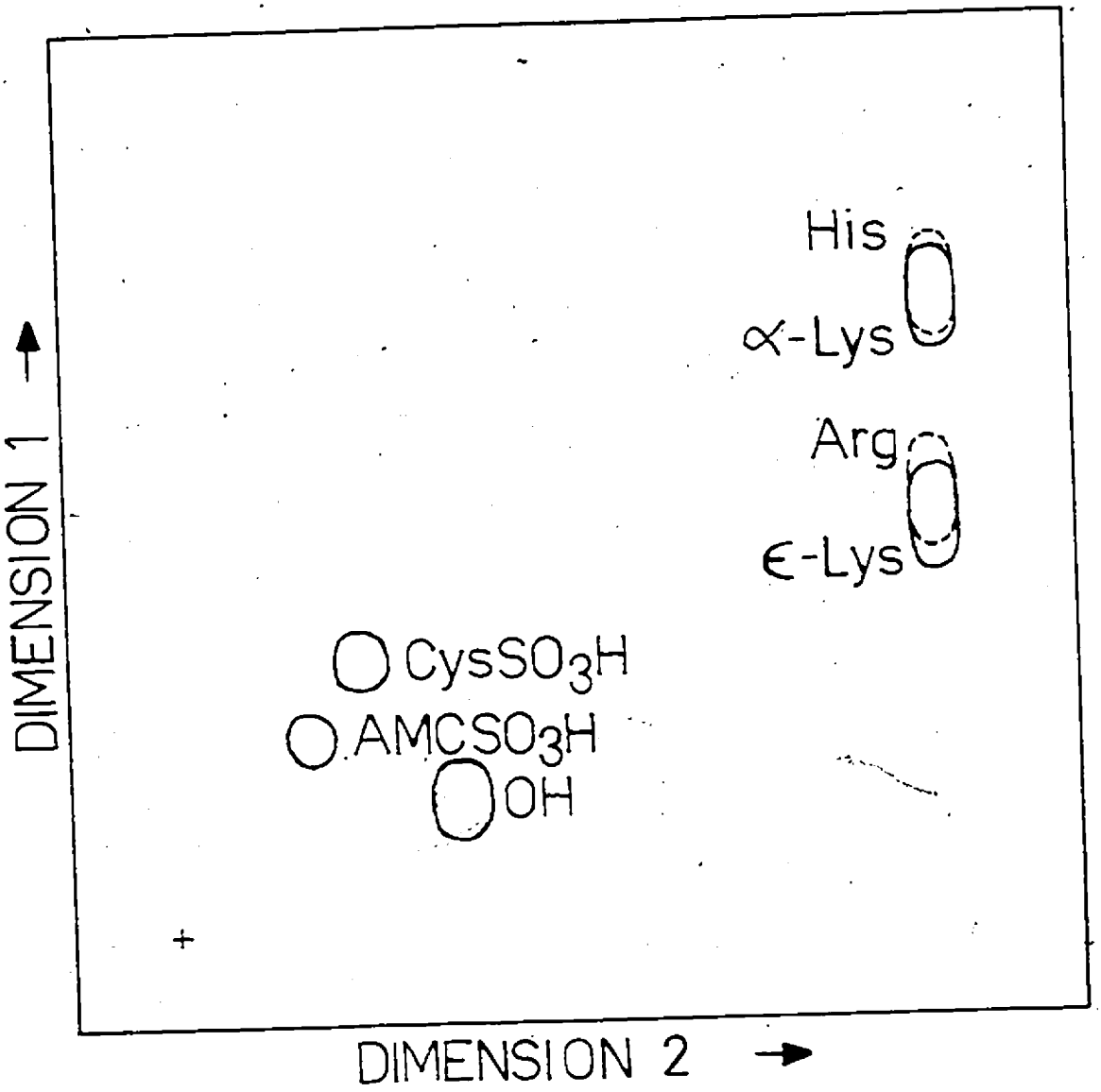


FIGURE 3

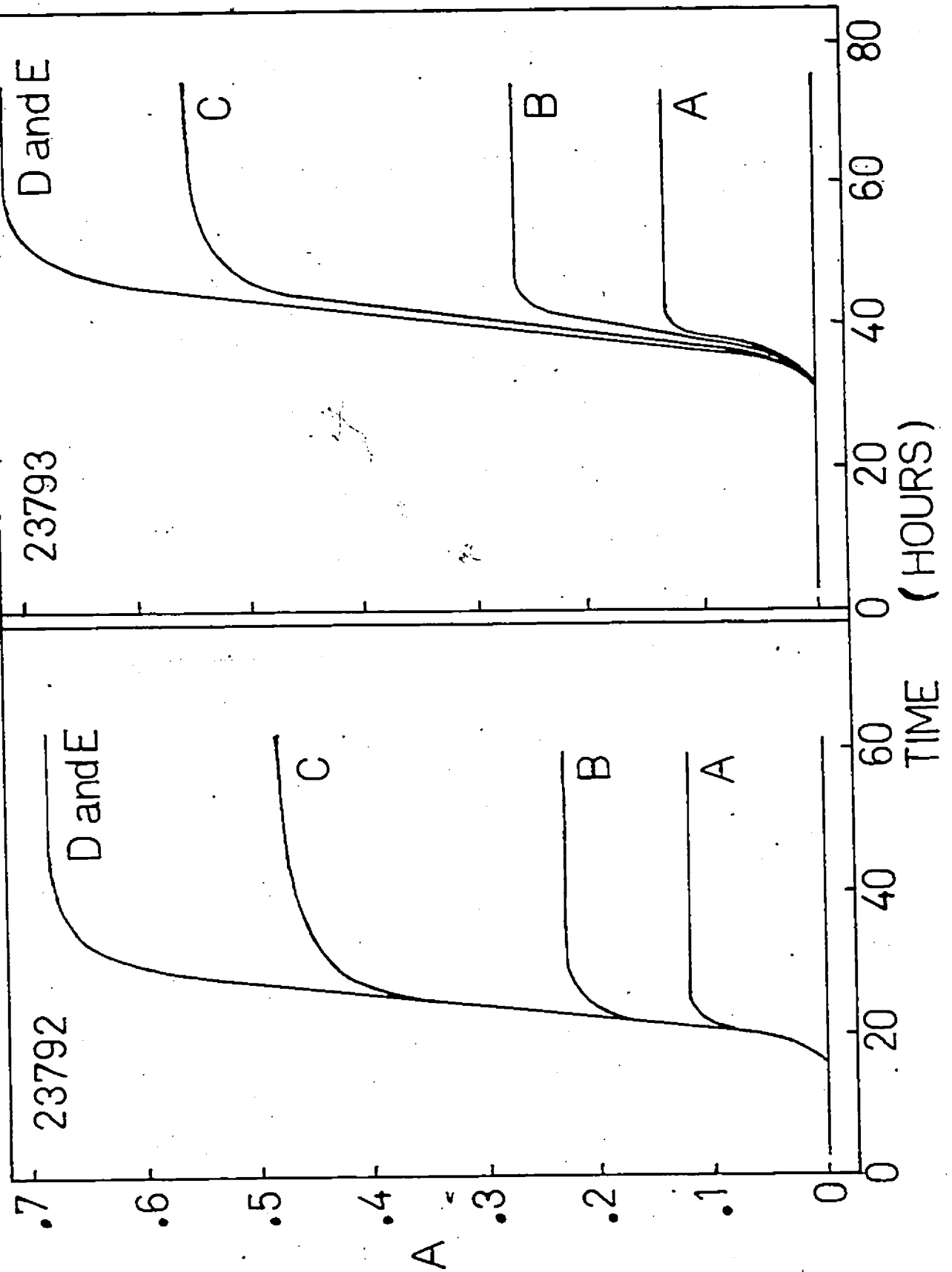
THE EFFECT OF INCREASING L-CYSTEINE CONCENTRATION ON THE GROWTH OF CYSTEINE-OBLIGATE *E. COLI*

Legend

Typical results for growth of *E. coli* ATCC 23792 and 23793 in Medium 2 containing increasing concentrations L-cysteine: 14, 28, 56, 280, 560 µM labelled A-E, respectively.

FIGURE 3

THE EFFECT OF INCREASING L-CYSTEINE CONCENTRATION ON THE GROWTH OF CYSTEINE-OBLIGATE E. COLI



value of A at the stationary phase) was directly proportional to the concentration of cysteine up to about 80 μM (A ≈ 0.65). No further increase in total growth was observed with higher concentrations of cysteine. Presumably one or more components in the basal medium became growth-limiting. The experiment was repeated several times and the final amount of growth, at each of the tested growth-limiting concentrations of cysteine was reproducible with a coefficient of variation less than 7%. It was assumed for further experiments that the amino acid composition of the medium was growth-limiting provided that the total growth corresponded to a stationary phase A value of less than 0.6.

Growth curves and amino acid uptake results were obtained for cultures of E. coli ATCC 23792 and 23893 grown in Medium 2 containing L-cysteine (28 or 56 μM), or both L-cysteine (28 or 56 μM) and AMC (185 μM), or only AMC (185 μM). A typical set of growth curves for each strain is shown in Figure 4. Both strains grew in the media containing cysteine or cysteine and AMC but not in the medium with AMC alone. At growth-limiting concentrations of cysteine (28 or 56 μM) when AMC was also present, a very slight, but reproducible, decrease in the lag phase and a significant, reproducible increase in the total growth were observed for ATCC 23792 and 23793. Amino acid uptake results for both strains grown on cysteine or cysteine and AMC, showed all of the cysteine was taken up. E. coli ATCC 23792 also took up all of the AMC. E. coli ATCC 23793 assimilated only about 40% of the AMC at 56 μM L-cysteine and only about 15% of the AMC at 28 uM L-cysteine.

DNS-cysteic acid, but no DNS-α-methyl-cysteic acid was detected

FIGURE 4

GROWTH RESPONSE OF CYSTEINE-OBLIGATE E. COLI

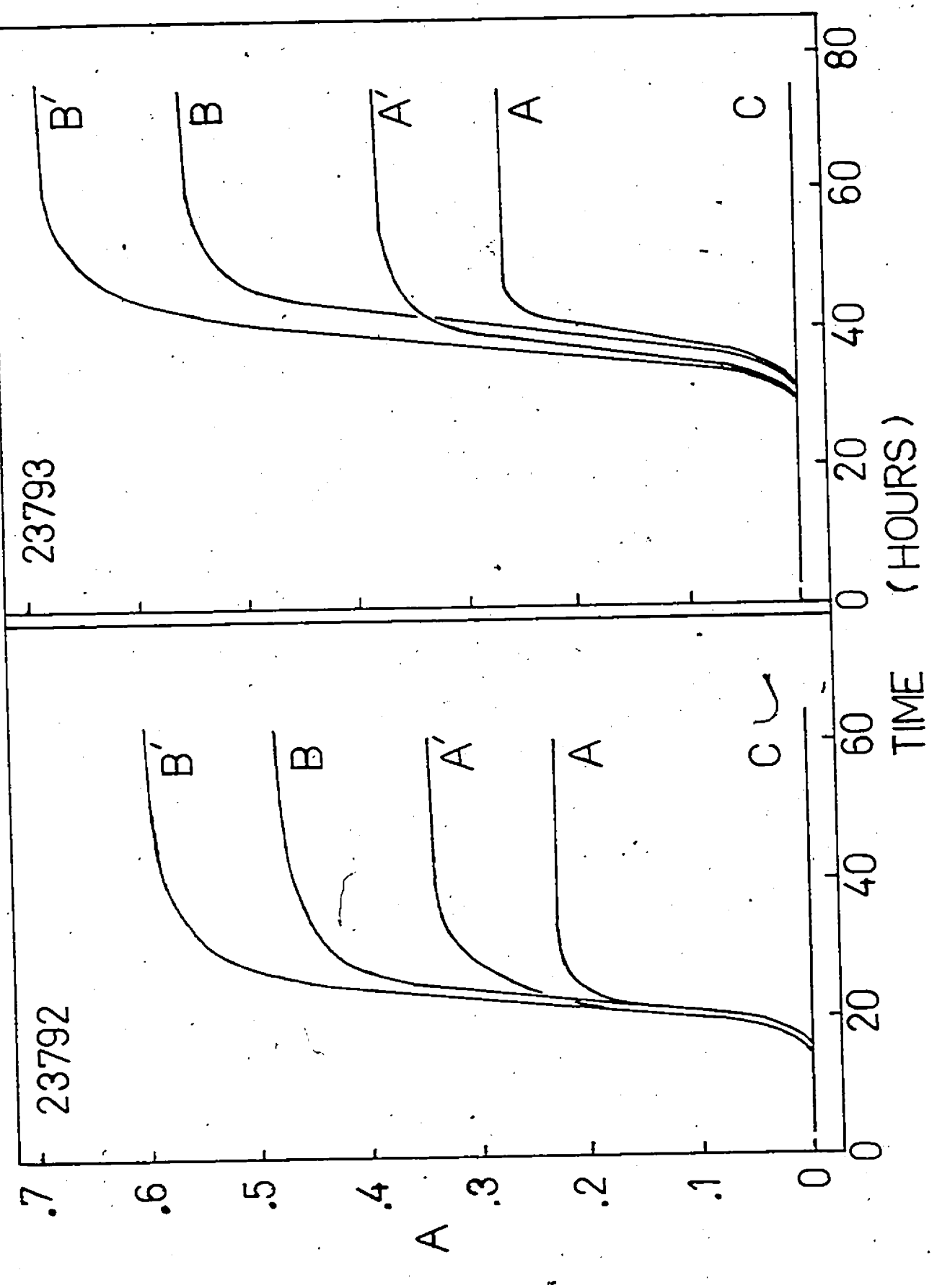
TO AMC

Legend

Typical results for growth of E. coli ATCC 23792 and 23793 in Medium 2 plus the following amino acids: A and A´, L-cysteine (28 µM); B and B´, L-cysteine (56 µM); A´ and B´, also contained AMC (185 µM); C, AMC (185 µM).

FIGURE 4

GROWTH RESPONSE OF CYSTEINE-OBLIGATE E. COLI TO AMC



on the chromatograms corresponding to the protein isolated from the cells of either strain in the presence of cysteine and AMC. Growth-limiting concentrations of L-cysteine were used in these experiments in order to detect the effect of AMC on total growth. Also, in the event that AMC was incorporated into protein, a high AMC to cysteine ratio would have minimized the competition between AMC and cysteine.

When the 'cysteine' required for protein biosynthesis could have been derived from the AMC present in the medium or from in vivo L-cysteine biosynthesis (E. coli ATCC 27257), the detection of only DNS-cysteic acid confirmed that the latter route was chosen. When AMC was the only possible source of 'cysteine', as for the cysteine-obligate E. coli ATCC 23792 and 23793 in Medium 2 containing only AMC, the cells did not grow. There are two possible explanations for this result. The first is that AMC was not an acceptable replacement for L-cysteine. Therefore, no protein was synthesized and consequently no growth occurred. The second is that as the cells added to the culture in the innoculum became metabolically active, AMC was a suitable source of 'cysteine' and protein was synthesized, but because of the presence of the analogue this protein was biologically inactive. Therefore, it was as if no protein was synthesized and consequently no growth occurred. If this latter hypothesis is true, cells in the presence of both AMC and L-cysteine should incorporate both amino acids into protein. Growth should occur because this protein should be somewhat active because of the reduced amount of analogue. In fact, growth did occur, but the detection of only DNS-cysteic acid disproves this second possibility. Therefore, protein

biosynthesis is sufficiently specific to prevent α-methyl-cysteine from being incorporated into protein.

The amino acid uptake analyses were necessary to dispel the possibility that AMC was not incorporated into protein because of a lack of AMC transport into the cell. Even though two E. coli strains, ATCC 27257 and 23793 did not take up all of the AMC from the growth medium, sufficient amounts were assimilated to have been detected on TLC, if the analogue had been incorporated into the protein.

The lower limit for the detection of a dansyl-amino acid on thin layer chromatography is approximately 10^{-10} moles (136). Approximate calculations showed that for DNS-cysteic acid which was easily identified on TLC, about 10^{-8} moles were present. If AMC was incorporated in an amount equal to 1% of the total cysteine, then 10^{-10} moles of DNS-α-methyl-cysteic acid would be present, i.e., an amount close to the lower limit of detection. Since the analogue was not detected, it could be argued that AMC accounted for as much as 1% of the total cysteine. However, this can hardly be termed significant and certainly this analogue is not suitable for use in studies to determine the role of cysteine in the conformation and biological activity of specific proteins.

The L-cysteine supplied in the media was oxidized to L-cystine over a period of a few hours. The cells have both enzymic (cysteine reductase) and non-enzymic methods for reduction of this cystine since it is cysteine which is the substrate for cysteinyl-tRNA synthetase. AMC is also reduced by these systems (133) and therefore, the lack of AMC incorporation was not due to the absence of α-methyl-L-cysteine.

Furthermore, the possibility that most or all of the AMC taken into the cell was degraded, is unlikely. Most of the enzymes for amino acid catabolism utilize the cofactor pyridoxal phosphate. The mechanism for reaction requires the presence of an α-hydrogen on the substrate molecule which α-methyl-cysteine obviously lacks, e.g. cysteine desulfhydrase is inactive against α-methyl-cysteine (129).

B. UTILIZATION OF AMC BY CYSTEINE-OBLIGATE E. COLI

Although AMC was not incorporated into protein, the results in Figure 4 showed that the presence of AMC in the media increased the total growth attained by these cysteine-obligate strains of E. coli grown in media where the amount of cysteine was otherwise growth-limiting. The following experiments were performed to provide an explanation for these results.

1. L-Methionine-Induced Growth Enhancement

In the absence of L-methionine, E. coli synthesize this amino acid from L-cysteine. When these strains of E. coli are grown in Medium 2 containing a growth-limiting concentration of L-cysteine, the sole or at least the major metabolic fate is incorporation into protein either as cysteine or methionine. The ratio of cysteine to methionine will be a constant (w) dependent on the requirements for these amino acids in the proteins of this strain. To determine the effect of the addition of methionine to the medium and to estimate the value of w for these strains of E. coli, a duplicate series of cultures of E. coli ATCC 23792 and 23793 were grown in Medium 2 containing a constant growth-limiting concentration of L-cysteine (14 μM) plus increasing concentrations of

L-methionine (0, 14, 28, 42, 56 μM). The value of A at the stationary phase (A_{sp}) was determined for each culture. The two values of A_{sp} at each concentration of methionine were averaged. The average A_{sp} ($\bar{A}_{sp}$) was plotted against the concentration of methionine (Figure 5).

The results were explained on the assumption that as the concentration of methionine was increased, more of the necessary methionine was derived from the medium and less cysteine was converted to methionine. Thus, the availability of both amino acids was increased, more protein could be synthesized and more growth was possible. Eventually all of the cysteine available for protein synthesis was utilized as cysteine and the corresponding necessary amount of methionine was derived from the medium. At this point, a maximum amount of growth (A_{max}) was observed. Further increases in the availability of methionine were of no consequence due to the lack of additional cysteine.

Therefore, the value of w for each strain was calculated by dividing the amount of cysteine available in the medium (1.4 μmoles) by the amount of methionine available in the medium when A_{max} was attained. For E. coli ATCC 23792 w was 0.36 (1.40 divided by 3.85), and for E. coli ATCC 23793 w was 0.42 (1.40 divided by 3.33). In other words for each 1 μmole of cysteine incorporated into protein, the number of μmoles of methionine incorporated were 2.75 and 2.39, respectively. Not only were these values comparable which one would expect of two closely related strains of E. coli, these values correlated well with the values obtained on amino acid analysis of the trichloroacetic acid-precipitable protein.

2. Combined AMC- and L-Methionine-Induced Growth Enhancement

FIGURE 5

L-METHIONINE-INDUCED GROWTH ENHANCEMENT

Legend

A duplicate series of cultures of E. coli ATCC 23792 and 23793 were grown in Medium 2 containing L-cysteine (14 µM) plus increasing concentrations of L-methionine (0, 14, 28, 42, 56 µM). Each value of $\bar{A}_{sp}$ is plotted against the corresponding L-methionine concentration.

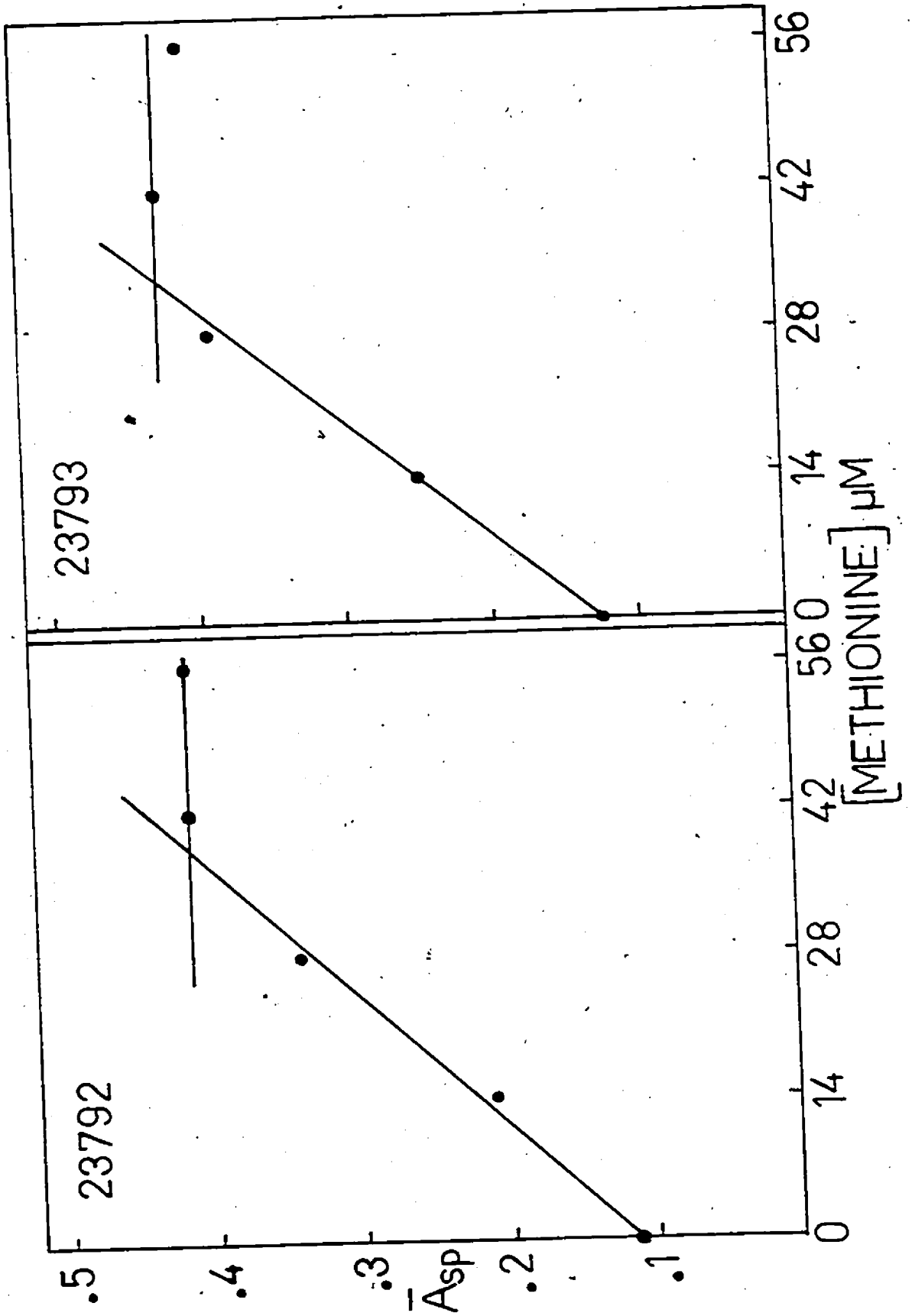


FIGURE 5

L-METHIONINE-INDUCED GROWTH ENHANCEMENT

Another duplicate series of cultures of each strain were grown in Medium 2 containing the same constant growth-limiting concentration of L-cysteine (14 μM) and the same concentrations of L-methionine (0, 14, 28, 42, 56 μM). However, each of these growth media also contained a constant concentration of AMC (185 μM). Again, the average of the two values of A_{sp} observed at each concentration of methionine was calculated. The $\bar{A}_{sp}$ was plotted against the concentration of methionine (Figure 6).

The A_{max} for E. coli ATCC 23792 was approximately the same as that observed in the absence of AMC and was attained at a lower initial methionine concentration. These observations were rationalized on the assumption that AMC and methionine enhanced growth via a common mechanism, i.e., the presence of either compound in the media represented a source of methionine other than that which could have been derived from cysteine. The A_{max} values, observed in the presence of AMC, were the same since A_{max} was reached once all the cysteine was utilized as cysteine and the corresponding necessary amount of methionine, irrespective of the source, was obtained from the medium. Since the amount of methionine in the medium necessary to achieve A_{max} was constant and because some of this methionine was derived from AMC, A_{max} was reached at a lower initial methionine concentration (c.f. 2.30 versus 3.85 in the absence of AMC).

There are two other possible fates of cysteine which have not yet been considered. It was possible in the presence of only cysteine, that a significant portion of it was catabolized or converted to compounds other than methionine which were also required for growth, but which were not supplied in the medium and which the cells did not synthesize from

FIGURE 6

AMC- AND L-METHIONINE INDUCED GROWTH ENHANCEMENT

Legend

A duplicate series of cultures of E. coli ATCC 23792 and 23793 were grown in Medium 2 containing L-cysteine (14 µM) and AMC (185 µM) plus increasing concentrations of L-methionine (0, 14, 28, 42, 56 M). Each value of $\bar{A}_{sp}$ is plotted against the corresponding L-methionine concentration. The dashed line represents the results in the absence of AMC (FIGURE 5).

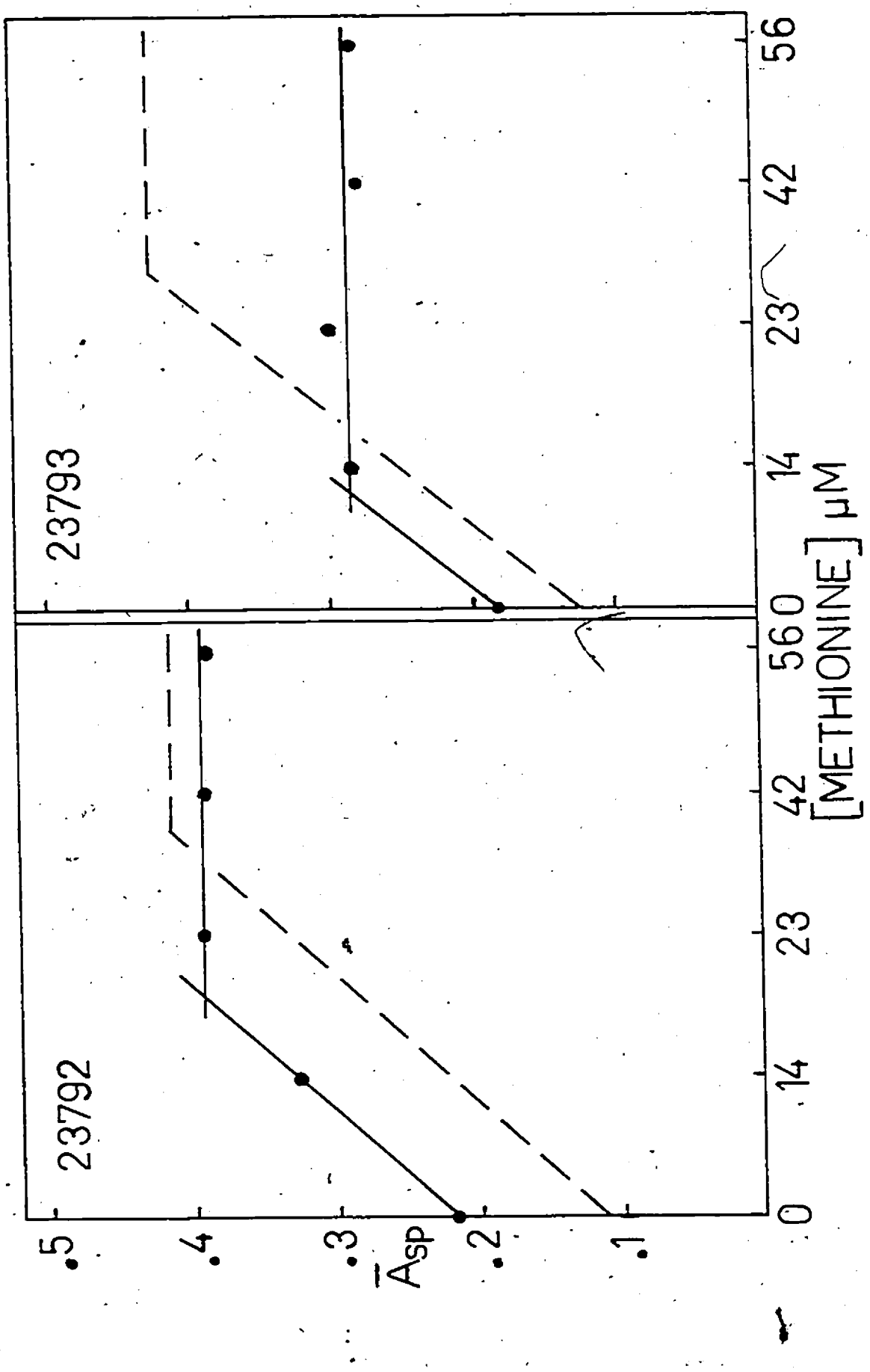


FIGURE 6

AMC- AND L-METHIONINE INDUCED GROWTH ENHANCEMENT

other components of the medium. Therefore, the growth enhancement due to AMC would be independent of the methionine-induced growth enhancement, i.e., AMC would spare cysteine either by serving as an alternate source of other necessary compounds normally derived from cysteine or by inhibiting cysteine catabolism. A plot such as that in Figure 6 would be expected to show a higher value of A_{max} at a greater methionine concentration. These changes would have occurred because a portion of cysteine formerly unavailable for protein synthesis, either as cysteine or methionine would have then become available. Since the ratio of cysteine to methionine incorporated into protein would still have been the same, more methionine could have been utilized. This increase in the amount of cysteine and utilizable methionine would have resulted in increased amounts of growth. These characteristics were not found. Therefore, the value of w, calculated on the assumption that no cysteine was converted to other compounds, was correct. Furthermore, AMC-induced growth enhancement was due to the conversion of AMC, like cysteine, to methionine.

The results for E. coli ATCC 23793 were not explainable in the same way and they will be considered later in this presentation.

3. AMC-Induced Growth Enhancement

A series of cultures of both cysteine-obligate E. coli strains were grown in Medium 2 containing a constant growth-limiting concentration of L-cysteine (28 μM) and increasing concentrations of AMC (0, 18.5, 37, 55.5, 74, 185, 370, 740 μM). The stationary phase A values were determined. The value of ΔA_a, where ΔA_a was the difference between the stationary phase A value at some concentration of AMC equal to "a" (A_a) and the stationary phase A value in the absence of AMC (A_{min}), was

plotted against the concentration of AMC (Figure 7). Each of the ΔA_a values plotted is actually the average of at least two determinations. The coefficient of variation of ΔA_a at each concentration of AMC was less than 8%.

For E. coli 23792 ΔA_a increased as the concentration of AMC, i.e., the availability of methionine, increased. The efficiency of AMC conversion to methionine was low. From the value calculated for the amount of AMC converted to methionine and utilized in protein (Chapter IV, equation 14) at each concentration of AMC, the percentages of AMC utilized were calculated. The values decreased from a maximum of about 12% at 18.5 μM AMC to a minimum of 3% at 740 μM AMC. This decrease may have resulted from an enzyme saturation phenomenon. Theoretically, the concentration of AMC would eventually be high enough so that all of the cysteine would be utilized as cysteine and all of the necessary methionine would be derived from AMC, i.e., A_{max} would be reached. This could not be proven experimentally because of the high AMC concentrations which would have been required. However, in the double reciprocal plot of ΔA_a^{-1} versus $[AMC]^{-1}$, the y-intercept value equalled the reciprocal of the theoretical ΔA_{max} which was 1.65 (A_{min} =0.220, ∴ theoretical A_{max} = 0.805, ∴ ΔA_{max} =0.605) (Figure 8).

A kinetic model based on the assumption that growth enhancement occurred due to the conversion of AMC to methionine has been developed. The final equation for this model states that:

$$\Delta A_a = wK\,[AMC]_t \ln\left(\frac{[cys]_t(w+1)}{[AMC]_t\,wK} + 1\right) \Big/ k(w+1)^2 \qquad \text{(equation 15)}$$

FIGURE 7

AMC-INDUCED GROWTH ENHANCEMENT

Legend

E. coli ATCC 23792 and 23793 were grown in Medium 2 containing L-cysteine (28 µM) and increasing concentrations of AMC (0, 18.5, 37, 55.5, 74, 185, 370, 740 µM). Each value of ΔA_a is plotted against the corresponding AMC concentration.

FIGURE 7

AMC-INDUCED GROWTH ENHANCEMENT

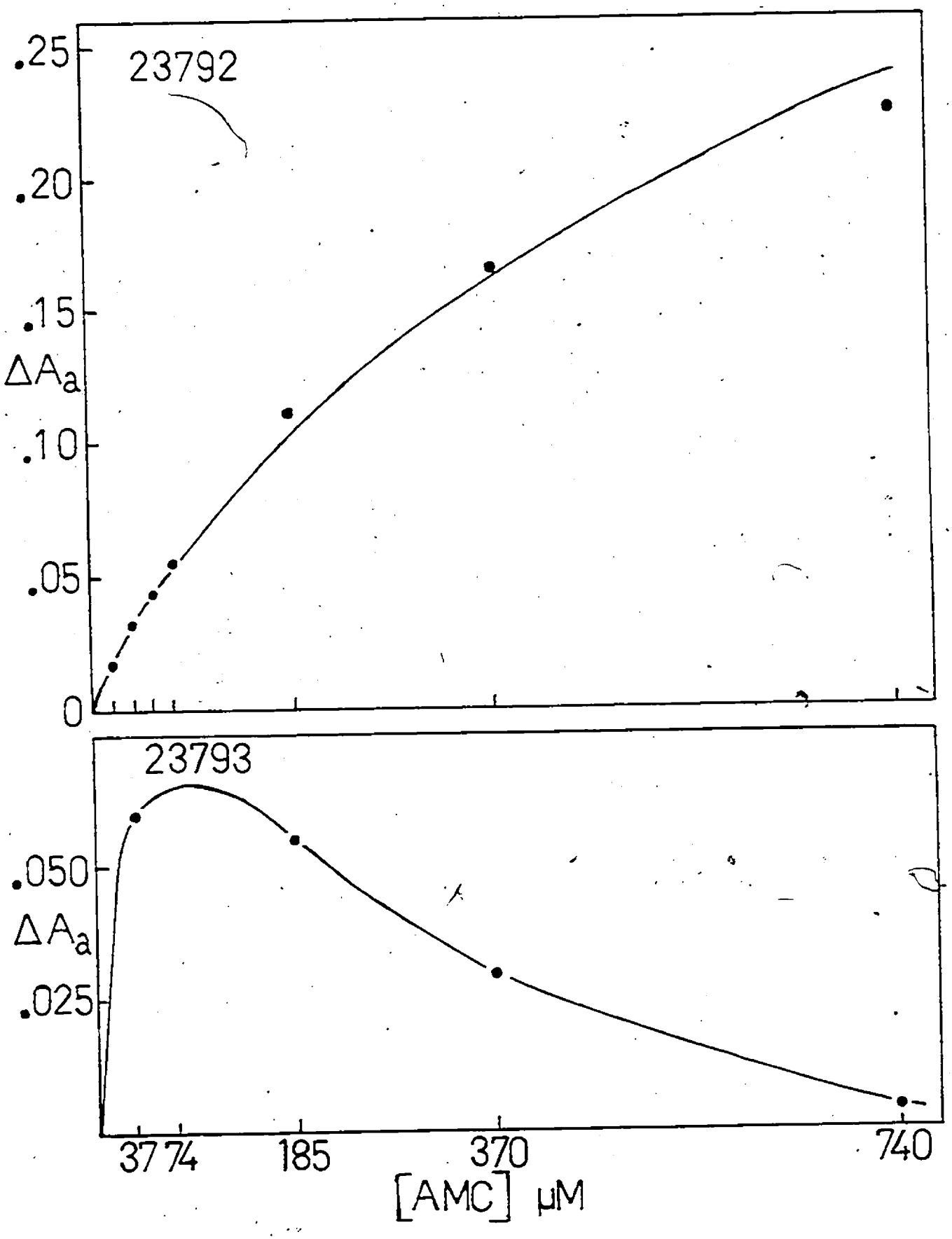


FIGURE 8

DOUBLE RECIPROCAL PLOT OF

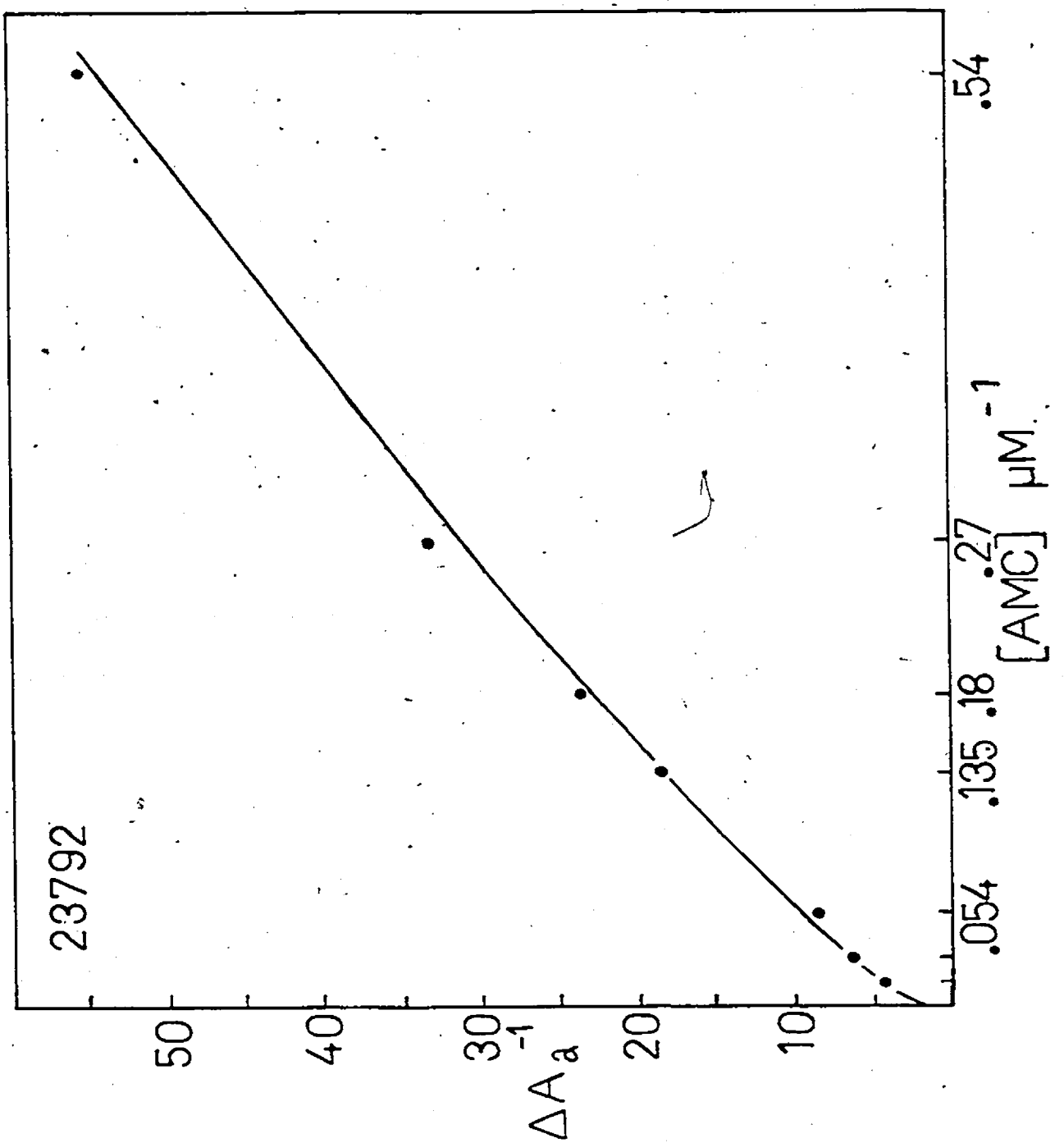
AMC-INDUCED GROWTH ENHANCEMENT

Legend

A double reciprocal plot of the results in FIGURE 7 for E.coli ATCC 23792.

FIGURE 8

DOUBLE RECIPROCAL PLOT OF AMC-INDUCED GROWTH ENHANCEMENT



where concentration terms represent the number of μmoles of the indicated amino acid originally present in the medium; k is the proportionality constant relating the amount of growth (A at the stationary phase) to the number of μmoles of cysteine used as such in protein synthesis; and K is a constant dependent on the enzymatic rate and dissociation constants of the rate-limiting step in the conversion of cysteine and AMC to methionine. The derivation of this equation is shown in Chapter IV. The value of k (3.4) was calculated using equation 12 (Chapter IV), where w was 0.36, $[cys]_t$ was 2.8 and A_{min} was the observed value of 0.220. A value of K was calculated for each of the various AMC concentrations using the corresponding observed value of ΔA_a and equation 15 (Chapter IV); where w was 0.36, k was 3.4 and $[cys]_t$ was 2.8. The mean value and standard deviation of K was 0.034 ± .003.

By substituting the calculated value of w (0.36), k (3.4) and K (0.034), a value of $[cys]_t$ equal to 2.8 and various concentrations of AMC into equation 15, curves were generated for ΔA_a versus $[AMC]_t$ and ΔA_a^{-1} versus $[AMC]_t^{-1}$. These curves were found to fit the experimental data and they are the curves drawn in Figure 7 (for ATCC 23792) and Figure 8.

The results for E. coli (ATCC 23793) (Figures 6 and 7) did not show conclusively that growth enhancement in the presence of AMC was due to the conversion of this analogue to methionine. Figure 7 shows growth enhancement at low concentrations of AMC and Figure 6 shows that at 14 μM methionine and 185 μM AMC there was growth enhancement above that due to the methionine. Growth enhancement was presumed to be due to the conversion of AMC to methionine since the same metabolic pathway is

responsible for the biosynthesis of methionine in both strains. However, growth enhancement decreased at high concentrations of AMC (Figure 7). Also, the maximum amount of growth attained due to an ample supply of methionine (Figure 5) was not observed when both a growth-enhancing concentration of AMC (185 μM) and excess methionine were present in the medium (Figure 6). Whether these observations were the result of a transport problem or a metabolic regulatory or inhibitory phenomenon has not as yet been determined.

CHAPTER IV

MATHEMATICAL MODEL FOR THE UTILIZATION OF AMC BY CYSTEINE-OBLIGATE E. COLI

The production of methionine for protein synthesis is assumed to result from the conversion of AMC or cysteine to methionine by the same metabolic pathway. The overall conversion will be governed by the rate-limiting enzymatic reaction in the pathway and may be represented by:

$$cys + E \underset{}{\overset{K_2}{\rightleftharpoons}} E\ cys \xrightarrow{k_2} cys_{mp}$$

$$AMC + E \underset{}{\overset{K_3}{\rightleftharpoons}} E\ AMC \xrightarrow{k_3} AMC_{mp}$$

where E = rate-limiting enzyme; cys = cysteine; AMC = α-methyl-DL-cysteine; cys_{mp} = methionine produced from cysteine and incorporated into protein; and AMC_{mp} = methionine produced from AMC and incorporated into protein.

At any time during the growth period:

$$\frac{d\ [AMC]_{mp}}{dt} = k_3[E \cdot AMC] = \frac{k_3}{K_3}\ [AMC]\ [E] \qquad (1)$$

$$\frac{d\ [cys]_{mp}}{dt} = k_2[E \cdot cys] = \frac{k_2}{K_2}\ [cys]\ [E] \qquad (2)$$

Dividing (1) by (2)

$$\frac{d\ [AMC]_{mp}}{d\ [cys]_{mp}} = \frac{k_3}{K_3}\ \frac{K_2}{k_2}\ \frac{[AMC]}{[cys]} \qquad (3)$$

The amount of free AMC at any time during the growth period is given by: $[AMC] = [AMC]_t - [AMC]_{mp}$, where $[AMC]_t$ = total amount of AMC originally in the medium;

but since $[AMC]_{mp}$ is small compared to $[AMC]_t$, it is assumed that

$$[AMC] = [AMC]_t - [AMC]_{mp} \approx [AMC]_t \quad (4)$$

The amount of free cysteine at any time during the growth period is given by:

$$[cys] = [cys]_t - [cys]_{mp} - [cys]_p \quad (5)$$

where $[cys]_t$ = amount of cysteine originally in the medium; and $[cys]_p$ = amount of cysteine incorporated into protein

but $[cys]_p = w\ [meth]_p \quad (6)$

where w = the constant for the ratio of cysteine to methionine incorporated into protein; and $[meth]_p$ = total amount of methionine produced from both sources and incorporated into protein

∴ substituting (6), (5) becomes

$$[cys] = [cys]_t - [cys]_{mp} - w\ [meth]_p \quad (5a)$$

$$\therefore [meth]_p = [AMC]_{mp} + [cys]_{mp} \quad (7)$$

∴ substituting (7), (5a) becomes

$$[cys] = [cys]_t - [cys]_{mp} - w\ [cys]_{mp} - w\ [AMC]_{mp} \quad (5b)$$

rearranging

$$[cys] = [cys]_t - (w+1)\ [cys]_{mp} - w\ [AMC]_{mp} \quad (8)$$

∴ substituting (4) and (8), (3) becomes

$$\frac{d\ [AMC]_{mp}}{d\ [cys]_{mp}} = \frac{K\ [AMC]_t}{[cys]_t - (w+1)\ [cys]_{mp} - w[AMC]_{mp}} \quad (9)$$

where $K = \frac{k_3}{K_3}\frac{K_2}{k_2}$

rearranging

$$\left([cys]_t - (w+1)[cys]_{mp} - w[AMC]_{mp}\right) d[AMC]_{mp} - \left(K[AMC]_t\right) d[cys]_{mp} = 0 \quad (9a)$$

solution of the differential equation (9a)

Is the differential equation exact?

let $x = [AMC]_{mp}$; $y = [cys]_{mp}$

$$M = [cys]_t - (w+1)[cys]_{mp} - w[AMC]_{mp}$$

$$N = -K[AMC]_t$$

∴ simply, (9a) can be represented by

$$Mdx + Ndy = 0$$

$$\therefore \frac{\delta M}{\delta y} = -(w+1) \qquad \frac{\delta N}{\delta x} = 0 \quad \text{Since } \frac{\delta M}{\delta y} \neq \frac{\delta N}{\delta x} \quad \therefore \text{ not exact}$$

$$\therefore \frac{\frac{\delta M}{\delta y} - \frac{\delta N}{\delta x}}{N} = \frac{-(w+1) - 0}{-K[AMC]_t} = \frac{(w+1)}{K[AMC]_t}$$

$$\therefore \text{integration constant} = \mu = e^{\int \frac{(w+1)}{K[AMC]_t} d[AMC]_{mp}} = e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}}$$

multiplying M and N by the integration constant, the resulting differential equation is exact

$$\left([cys]_t - (w+1)[cys]_{mp} - w[AMC]_{mp}\right) e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}} d[AMC]_{mp}$$

$$- K[AMC]_t \, e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}} d[cys]_{mp} = 0 \quad (9b)$$

$\therefore \mu M dx + \mu N dy = 0$ is exact, then there exists a function $U(x,y)$ such that

$$\mu M dx + \mu N dy = dU$$

but then $$dU = \frac{\delta U}{\delta x} dx + \frac{\delta U}{\delta y} dy$$

$$\therefore \quad \frac{\delta U}{\delta x} = \mu M \qquad \frac{\delta U}{\delta y} = \mu N$$

$$\therefore \left([cys]_t - (w+1)[cys]_{mp} - w[AMC]_{mp}\right) e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}} d[AMC]_{mp}$$

$$- K[AMC]_t e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_{mp}}} d[cys]_{mp} = dU$$

$$\text{and } \frac{\delta U}{\delta [AMC]_{mp}} = \left([cys]_t - (w+1)[cys]_{mp} - w[AMC]_{mp}\right) e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}} \quad \text{(i)}$$

$$\frac{\delta U}{\delta [cys]_{mp}} = - K[AMC]_t e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}} \quad \text{(ii)}$$

$\therefore$ to solve the exact differential equation, need only to determine $U(x,y)$

from (i) can find U by the reverse differentiation with respect to x, i.e., integration with respect to x, keeping y constant

the constant of integration which must be added is independent of x but might depend on y; i.e. the arbitrary constant may really be a function of y,

and this is denoted by f(y).

$$U = \int \frac{\delta U}{\delta x}\, \delta x + f(y)$$

$$U = \int \frac{\delta U}{\delta [AMC]_{mp}}\, \delta [AMC]_{mp} + f\left([cys]_{mp}\right)$$

$$U = \int \left([cys]_t - (w+1)[cys]_{mp} - w[AMC]_{mp}\right) e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}}\, \delta [AMC]_{mp} + f\left([cys]_{mp}\right)$$

$$U = \frac{[cys]_t}{\frac{(w+1)}{K[AMC]_t}}\, e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}} - \frac{(w+1)[cys]_{mp}}{\frac{(w+1)}{K[AMC]_t}}\, e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}}$$

$$- \frac{w}{\left[\frac{(w+1)}{K[AMC]_t}\right]^2}\, e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}} \left(\frac{(w+1)[AMC]_{mp}}{k[AMC]_t} - 1\right) + f\left([cys]_{mp}\right)$$

$$U = \frac{e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}}}{\frac{(w+1)}{K[AMC]_t}} \left([cys]_t - (w+1)[cys]_{mp} - w[AMC]_{mp} + \frac{w}{\frac{(w+1)}{K[AMC]_t}}\right) + f\left([cys]_{mp}\right)$$

to find f(y), evaluate $\frac{\delta U}{\delta y}$

$$\frac{\delta}{\delta [cys]_{mp}} \left[\frac{e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}}}{\frac{(w+1)}{K[AMC]_t}} \left([cys]_t - (w+1)[cys]_{mp} - w[AMC]_{mp} + \frac{w}{\frac{(w+1)}{K[AMC]_t}} \right) + f([cys]_{mp}) \right]$$

$$= -\frac{(w+1)}{\frac{(w+1)}{K[AMC]_t}} e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}} + f'([cys]_{mp})$$

where $f'([cys]_{mp})$ = derivative of $f([cys]_{mp})$ with respect to $[cys]_{mp}$

$\therefore \frac{\delta U}{\delta y} = \mu N$

$\therefore$ from (ii)

$$-\frac{(w+1)}{\frac{(w+1)}{K[AMC]_t}} e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}} + f'([cys]_{mp}) = -K[AMC]_t e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}}$$

$\therefore f'([cys]_{mp}) = 0 \quad \therefore f([cys]_{mp}) = \text{constant} = C_1$

$$\therefore U = \frac{e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}}}{\frac{(w+1)}{K[AMC]_t}} \left([cys]_t - (w+1)[cys]_{mp} - w[AMC]_{mp} + \frac{w}{\frac{(w+1)}{K[AMC]_t}} \right) + C_1$$

thus the differential equation can be written

$$dU = 0$$

$$\therefore d\left[\frac{e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}}}{e^{\frac{(w+1)}{K[AMC]_t}}}\left([cys]_t-(w+1)[cys]_{mp}-w[AMC]_{mp}+\frac{w}{\frac{(w+1)}{K[AMC]_t}}\right)+C_1\right]=0$$

and integration yields

$$\frac{e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}}}{e^{\frac{(w+1)}{K[AMC]}}}\left([cys]_t-(w+1)[cys]_{mp}-w[AMC]_{mp}+\frac{w}{\frac{(w+1)}{K[AMC]_t}}\right)+C_1=C_2$$

or

$$\frac{e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}}}{e^{\frac{(w+1)}{K[AMC]_t}}}\left([cys]_t-(w+1)[cys]_{mp}-w[AMC]_{mp}+\frac{w}{\frac{(w+1)}{K[AMC]_t}}\right)=C$$

initially $[cys]_{mp} = [AMC]_{mp} = 0$

to evaluate C, substitute $[cys]_{mp} = [AMC]_{mp} = 0$

$$\therefore \frac{1}{\frac{(w+1)}{K[AMC]_t}}\left([cys]_t+\frac{w}{\frac{(w+1)}{K[AMC]_t}}\right)=C$$

$$\therefore \frac{e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}}}{e^{\frac{(w+1)}{K[AMC]_t}}}\left([cys]_t-(w+1)[cys]_{mp}-w[AMC]_{mp}+\frac{w}{\frac{(w+1)}{K[AMC]_t}}\right)$$

$$=\frac{1}{\frac{(w+1)}{K[AMC]_t}}\left([cys]_t+\frac{w}{\frac{(w+1)}{K[AMC]_t}}\right)$$

simplifying and rearranging, gives

$$[cys]_t-(w+1)[cys]_{mp}-w[AMC]_{mp}+\frac{w}{\frac{(w+1)}{K[AMC]_t}}=\frac{[cys]_t+\frac{w}{\frac{(w+1)}{K[AMC]_t}}}{e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}}} \quad (10)$$

which is the solution of the differential equation

$$\frac{d[AMC]_{mp}}{d[cys]_{mp}}=\frac{K[AMC]_t}{[cys]_t-(w+1)[cys]_{mp}-w[AMC]_{mp}} \quad (9)$$

check: rearrangement of (10) gives

$$[cys]_{mp}=\left[\frac{1}{(w+1)}\right]\left[[cys]_t-w[AMC]_{mp}+\frac{w\,K[AMC]_t}{(w+1)}-\frac{[cys]_t+\frac{w\,K[AMC]_t}{(w+1)}}{e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}}}\right] \quad (10a)$$

differentiating with respect to $[AMC]_{mp}$ gives

$$\frac{d[cys]_{mp}}{d[AMC]_{mp}}=\left[\frac{1}{(w+1)}\right]\left[-w-\left(\frac{\left([cys]_t+\frac{w\,K[AMC]_t}{w+1}\right)\left(\frac{(w+1)}{K[AMC]_t}\right)}{e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}}}\right)\right]$$

$$=\left[\frac{1}{(w+1)}\right]\left[-w-\left(\frac{-\frac{(w+1)[cys]_t}{K[AMC]_t}-w}{e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}}}\right)\right]$$

$$\frac{d[cys]_{mp}}{d[AMC]_{mp}} = \left[\frac{1}{w+1}\right]\left[-w + \frac{\frac{(w+1)[cys]_t}{K[AMC]_t} + w}{e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}}}\right] \qquad (10b)$$

inverting (9) and substituting for $[cys]_{mp}$ from (10a)

$$\frac{d[cys]_{mp}}{d[AMC]_{mp}} = \frac{[cys]_t - [cys]_t + w[AMC]_{mp} - \frac{wK[AMC]_t}{(w+1)} + \frac{[cys]_t + \frac{wK[AMC]_t}{(w+1)}}{e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}}}}{K[AMC]_t}$$

$$\frac{d[cys]_{mp}}{d[AMC]_{mp}} = \frac{-\frac{wK[AMC]_t}{(w+1)} + \frac{[cys]_t + \frac{wK[AMC]_t}{(w+1)}}{e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}}}}{K[AMC]_t}$$

$$= -\frac{w}{(w+1)} + \frac{\frac{(w+1)[cys]_t + wK[AMC]_t}{(w+1)K[AMC]_t}}{e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}}}$$

$$= \left[\frac{1}{w+1}\right]\left[-w + \frac{\frac{(w+1)[cys]_t}{K[AMC]_t} + w}{e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}}}\right] \equiv (10b)$$

Since the concentration of cysteine is growth-limiting, at the end of growth the amount of free cysteine is zero, i.e., (5) = (8) = 0 or that portion of (10) given by:

$$[cys]_t - (w+1)[cys]_{mp} - w[AMC]_{mp}$$

becomes zero. Therefore, at the end of growth, (10) is reduced and on rearrangement becomes

$$\frac{\frac{w}{(w+1)}}{K[AMC]_t} = \frac{[cys]_t + \frac{\frac{w}{(w+1)}}{K[AMC]_t}}{e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}}}$$

rearranging

$$e^{\frac{(w+1)[AMC]_{mp}}{K[AMC]_t}} = \frac{[cys]_t(w+1)}{w\,K[AMC]_t} + 1$$

$$\therefore \frac{(w+1)[AMC]_{mp}}{K[AMC]_t} = \ln\left(\frac{[cys]_t(w+1)}{w\,K[AMC]_t} + 1\right)$$

$$\therefore [AMC]_{mp} = \frac{K[AMC]_t \ln \frac{[cys]_t(w+1)}{w\,K[AMC]_t} + 1}{(w+1)} \qquad (11)$$

Since the concentration of cysteine is growth-limiting, at the end of growth all of the cysteine is used as cys_p or cys_{mp}

$\therefore [cys]_t = [cys]_p + [cys]_{mp}$

in the absence of AMC $\quad [cys]_{mp} = [meth]_p = \frac{[cys]_p}{w}$

$$\therefore [cys]_t = [cys]_p + \frac{[cys]_p}{w} = [cys]_p\left(1 + \frac{1}{w}\right) = [cys]_p\left(\frac{(w+1)}{w}\right)$$

$$[cys]_p = \frac{[cys]_t}{\frac{(w+1)}{w}}$$

in the presence of AMC $\quad cys_{mp} = [meth]_p - [AMC]_{mp} = \frac{[cys]_p}{w} - [AMC]_{mp}$

$$\therefore [cys]_t = [cys]_p + \frac{[cys]_p}{w} - [AMC]_{mp}$$

$$= [cys]_p\left(1+\frac{1}{w}\right) - [AMC]_{mp} = [cys]_p\left(\frac{(w+1)}{w}\right) - [AMC]_{mp}$$

$$[cys]_p = \frac{[cys]_t + [AMC]_{mp}}{\frac{(w+1)}{w}}$$

in both cases, the total amount of growth at the stationary phase (A) is proportional to the growth-limiting concentration of cysteine available as such for incorporation into protein, i.e., $[cys]_p$

$\therefore$ the minimum amount of growth attainable in the absence of AMC is

$$kA_{min} = [cys]_p = \frac{[cys]_t}{\frac{(w+1)}{w}} \tag{12}$$

where k is the proportionality constant

and the amount of growth attainable in the presence of AMC at some concentration "a" is

$$kA_a = [cys]_p = \frac{[cys]_t + [AMC]_{mp}}{\frac{(w+1)}{w}} \tag{13}$$

$\therefore$ the increase in growth due to the presence of AMC is

$$\Delta A_a = A_a - A_{min}$$

$$\Delta A_a = \frac{[cys]_t + [AMC]_{mp}}{k\,\frac{(w+1)}{w}} - \frac{[cys]_t}{k\,\frac{(w+1)}{w}}$$

$$\Delta A_a = \frac{[AMC]_{mp}}{k\,\frac{(w+1)}{w}} \tag{14}$$

substituting (11) for $[AMC]_{mp}$, (14) becomes

$$\Delta A_a = \frac{w\ K\ [AMC]_t \left(\ln \frac{[cys]_t (w+1)}{w\ K\ [AMC]_t} + 1\right)}{k\ (w+1)^2} \tag{15}$$

At a sufficiently large concentration of AMC, all of the methionine required will be derived from AMC and all the cysteine will be used as cysteine in protein synthesis and A_{max} will be attained

$\therefore$ $kA_{max} = [cys]_p = [cys]_t$

$\therefore$ $\Delta A_{max} = A_{max} - A_{min}$

$$\Delta A_{max} = \frac{[cys]_t}{k} - \frac{[cys]_t}{k\,\frac{(w+1)}{w}}$$

$$\Delta A_{max} = \frac{[cys]_t}{k(w+1)} \qquad (16)$$

At large $[AMC]_t$, (15) reduced to (16)

represent $\ln\left(\frac{[cys]_t(w+1)}{w\ K\ [AMC]_t} + 1\right)$ as $\ln\ (x + 1)$

if x is small (as it is when $[AMC]_t$ is large) then

$\ln\ (x + 1) \tilde{\sim} x$

(15) becomes

$$\Delta A_{max} = \frac{w\ K\ [AMC]_t\left(\frac{[cys]_t(w+1)}{w\ K\ [AMC]_t}\right)}{k\ (w+1)^2}$$

$$\Delta A_{max} = \frac{[cys]_t}{k(w+1)}$$

CHAPTER V

SUMMARY AND CONCLUSIONS

α-Methyl-cysteine was not incorporated into protein biosynthesized *in vivo* by either the methionine - or cysteine-obligate strains of *E. coli*. The translation process of protein biosynthesis is highly specific (10, 11). For several reasons, it seems most probable that the non-incorporation of this analogue resulted from the failure of cysteinyl-tRNA synthetase to activate this analogue or to transfer the activated analogue to cysteinyl-tRNA. Generally, the high degree of specificity exhibited by the aminoacyl-tRNA synthetases is thought to be of primary importance in minimizing errors in the translation process of protein biosynthesis. More specifically, cysteinyl-tRNA synthetase has been found to be highly specific in that S-substituted cysteine analogues were not activated by this enzyme (75-77). Also, the reports of work with other α-methyl-substituted amino acids showed that the analogue was not bound to the appropriate tRNA: α-methyl-DL-methionine was activated but not transferred to tRNA (71); and α-methyl-DL-tryptophan was not even activated (81). Since the α-H atom is not required from a mechanistic point of view for either activation or transfer to tRNA of natural amino acids, suggests that steric factors prohibit activation and/or transport to tRNA of those α-CH_3-containing amino acid analogues.

Assuming that α-methyl-cysteinyl-$tRNA^{Cys}$ is formed, it is difficult to suggest concrete reasons for its non-incorporation with protein. The EF-T_u · GTP complex appears to require an unaltered amino group on the aminoacyl moiety in order to direct the binding of the aminoacyl

-tRNA to mRNA (63-65). The actual binding of the anticodon of the tRNA moiety to the mRNA codon requires only mutual recognition of the two complementary triplet nucleotide sequences. Formation of a peptide bond again, obviously, requires the presence of a free amino group on the aminoacyl moiety. All of these conditions are satisfied by α-methyl-cysteinyl-tRNA^{Cys}. It is possible that the presence of the methyl group on the carbon atom also bearing the $-NH_2$ and -COOH groups adversely affects the steric requirements for binding of the aminoacyl-tRNA to the EF-T_u · GTP complex or peptide bond formation by peptidyl transferase. Furthermore, it is deemed unlikely that the incorporation of the analogue is blocked at the stage of peptide bond formation. If this were true, polypeptide chain biosynthesis would be terminated, with subsequent release of the incomplete polypeptide, i.e., an inhibition of 'complete' protein biosynthesis. It would be expected that such an occurrence would inhibit the growth of E. coli in the presence of AMC. No such observation was made; in fact, growth was stimulated and enhanced in the presence of the analogue.

This study provides the raison d'être for an in vitro analysis of the specificity of cysteinyl-tRNA synthetase towards AMC. Suitable subsequent studies concerned with the later stages of protein biosynthesis would be in order if α-methyl-cysteinyl-tRNA^{Cys} is observed.

The cysteine-obligate E. coli ATCC 23792 and 23793 were grown in a basal medium with cysteine as the sole amino acid supplement. Since cysteine and methionine are required for the biosynthesis of protein, some of the cysteine was incorporated directly into protein and the

remainder was incorporated indirectly, i.e., after conversion to methionine. The relative proportion of utilization as cysteine or methionine was dependent on the requirements for these amino acids in the protein of each strain. At sufficiently low concentrations of cysteine, the amount of growth at the stationary phase was directly proportional to the cysteine concentration. Increased growth was observed when additional quantities of either of these amino acids were supplied in the media. However, the addition of AMC was also observed to enhance growth. Since α-methyl-cysteine was not directly incorporated into protein, the experimental evidence led to the conclusion that AMC, like cysteine, was converted to methionine. By providing an alternate source of methionine, more cysteine was available for direct incorporation into protein. A mathematical model based on this interpretation appears to fit the experimental results.

The metabolic pathway for the biosynthesis of methionine from cysteine in E. coli is shown in Figure 9. The reduction of AMC to α-methyl-DL-cysteine by biochemical systems has been documented (129). Presumably, α-methyl-L-cysteine was converted to L-homocysteine by the action of cystathionine γ-synthetase and β-cystathionase. The generally accepted mechanisms (137) for these two pyridoxal-P-enzyme catalyzed reactions are shown in Figures 10 and 11, respectively. The synthesis of the α-methyl-substituted analogue of L-cystathionine (Figure 10) would not be expected to be such a problem since the α-methyl-substituted cysteine represents the attacking nucleophile and is not directly bound to the pyridoxal-P-enzyme complex. No information is available concerning

FIGURE 9

BIOSYNTHESIS OF METHIONINE

Legend

The biosynthetic pathway from L-cysteine and AMC leading to L-methionine in E. coli.

FIGURE 9

BIOSYNTHESIS OF METHIONINE

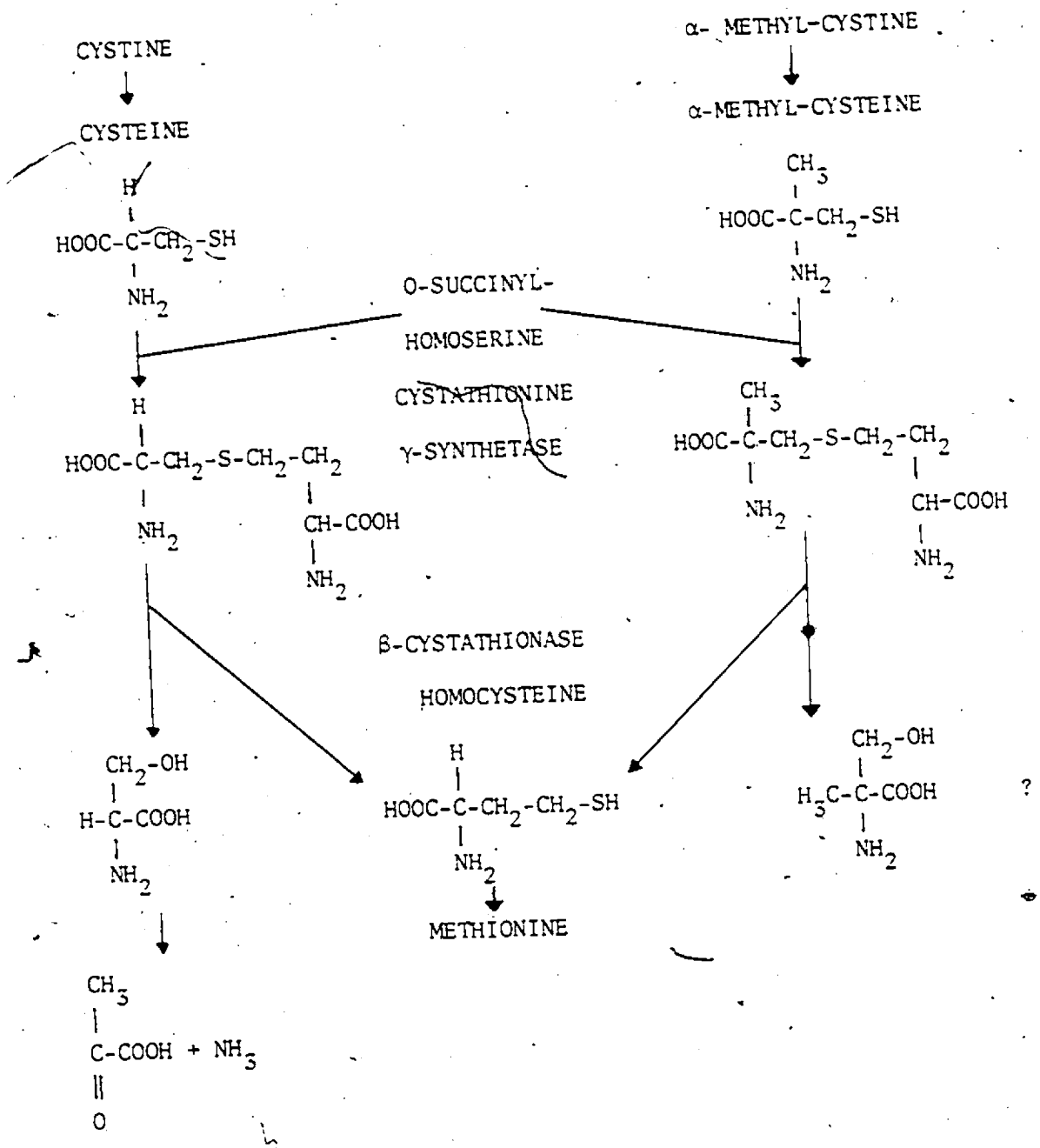


FIGURE 10

CYSTATHIONINE γ-SYNTHASE REACTION MECHANISM

Legend

See text.

FIGURE 10

CYSTATHIONINE γ-SYNTHASE REACTION MECHANISM

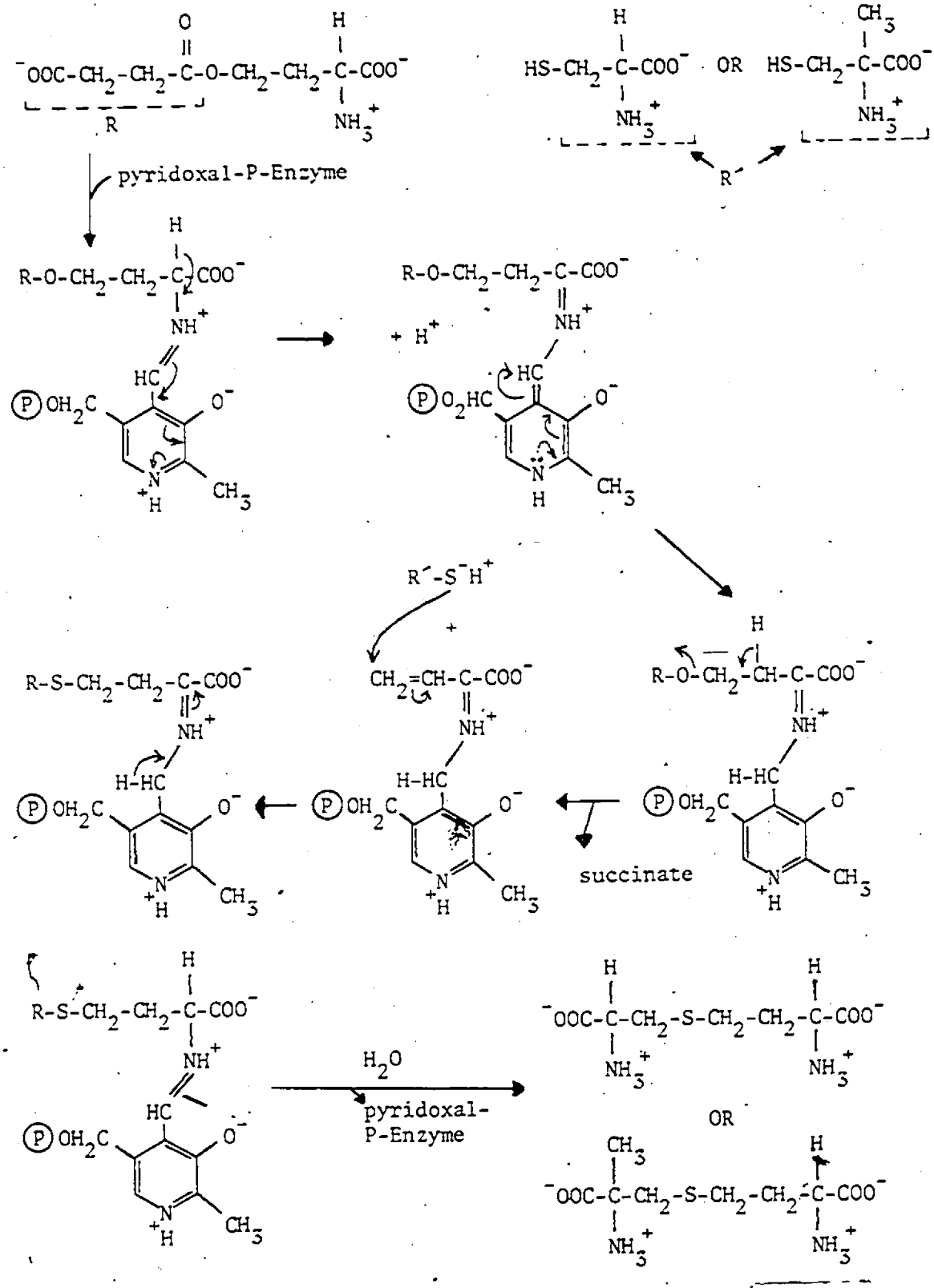


FIGURE 11

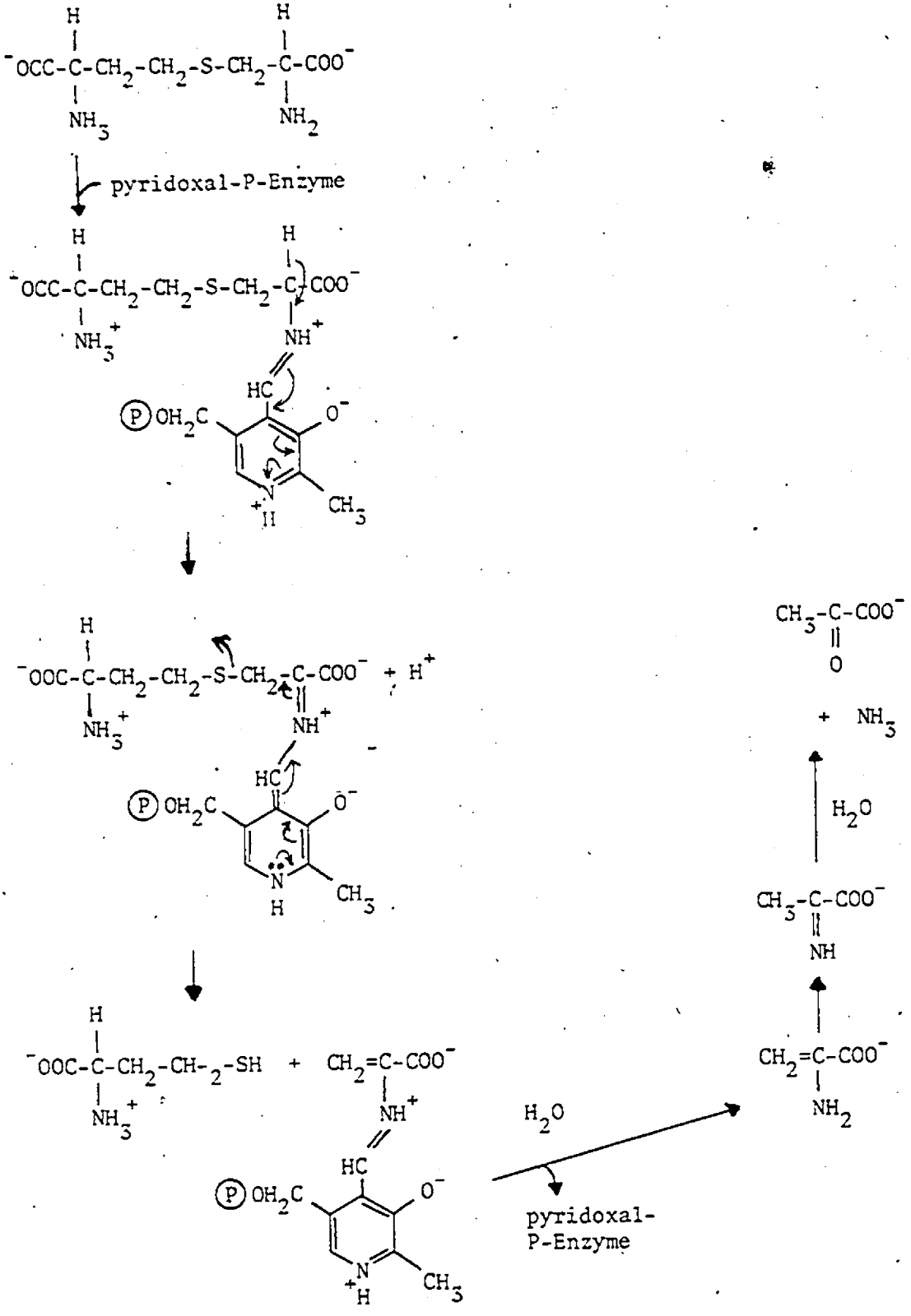
β-CYSTATHIONASE REACTION MECHANISM

Legend

See text.

FIGURE 11

β-CYSTATHIONASE REACTION MECHANISM



the possibility that some substituent(s) on the enzyme binds and directs the addition portion of this reaction. The cleavage of the α-methyl-substituted cystathionine analogue to produce homocysteine does present an interesting situation (Figure 11). After formation of the Schiff base between L-cystathionine and the pyridoxal-P-enzyme complex, the first step in the mechanism is the breaking of the C_α-H bond. In the analogue, however, the C_α-H bond is replaced with a C_α-CH_3 bond. The reaction can not proceed by the accepted mechanism. It is worthwhile to speculate what may be occurring, since the experimental data indicate that cleavage must have occurred in some fashion.

A variety of α-methyl-substituted analogues have been observed to undergo a variety of nonenzymatic pyridoxal catalyzed reactions (138-141). However, it has been shown that enzymatic pyridoxal-P catalyzed reactions are subject to substrate-, reaction- and stereospecificity (137). All enzymatic pyridoxal-P reactions proceed via the Schiff base formation between the amino group of the substrate and the aldehyde group of the pyridoxal-P. The second step is the breaking of one of the C_α bonds, either the C_α-H, C_α-$COO^\ominus$ or the C_α-R bond. Reaction specificity depends on the ability of the apoenzyme to bind the Schiff base in such a way that only one of the groups at the amino acid C_α is in a position to be lost. Since C_α is a chiral center, the dissymmetry of the apoenzyme easily allows a stereochemical differentiation between these groups. It is generally accepted that the C_α bond to be broken should lie in a plane perpendicular to the plane of the cofactor-imine pi system (142). The enzyme must control the conformation about the C_α-N bond if only a single group at C_α is to be labilized. It follows

that the binding sites for the R and COO^- groups of the amino acid substrate must have a specific dissymmetric relationship to the plane of the cofactor ring. While the above considerations are usually true, Bailey et al. (143, 144) have isolated a bacterial enzyme referred to as α-dialkylamino acid transaminase which transaminates L-alanine in the usual way with the C_α-H bond being labilized initially. However, the enzyme also catalyzes a decarboxylation-dependent transamination reaction - originally named by Kalyankar and Snell (140) - of dialkylamino acids (e.g. α-aminoisobutyrate) with the C_α-$COO^\ominus$ being labilized initially. Figure 12 shows the only possible mechanism for cleavage of the α-methyl-substituted analogue to produce homocysteine. The C_α-COO^- bond is labilized initially. The side products are of course different from those of the L-cystathionine reaction. Disregarding specificity, this mechanism is otherwise certainly feasible. Dunathan (137) stated that a change in the configuration at C_α will certainly destroy the positioning of the labilized group so that it is no longer labile. Furthermore, with a change of configuration, a new group at C_α may now achieve that geometry necessary for loss. Perhaps like the dialkylamino acid transaminase, changes in groups bonded to the C_α will influence the bond to be labilized. β-cystathionase does catalyze the β-elimination of a number of thioether, disulfide and hydroxyamino acids (144). Perhaps the cleavage of the α-methyl-substituted L-cystathionine analogue is mediated by another enzyme altogether. An in vitro study would be helpful in investigating the kinetic and mechanistic aspects of the enzymatic reactions which appear to catalyze the synthesis of L-homocysteine from AMC, i.e., cystathionine γ-synthase and β-cystathionase.

FIGURE 12

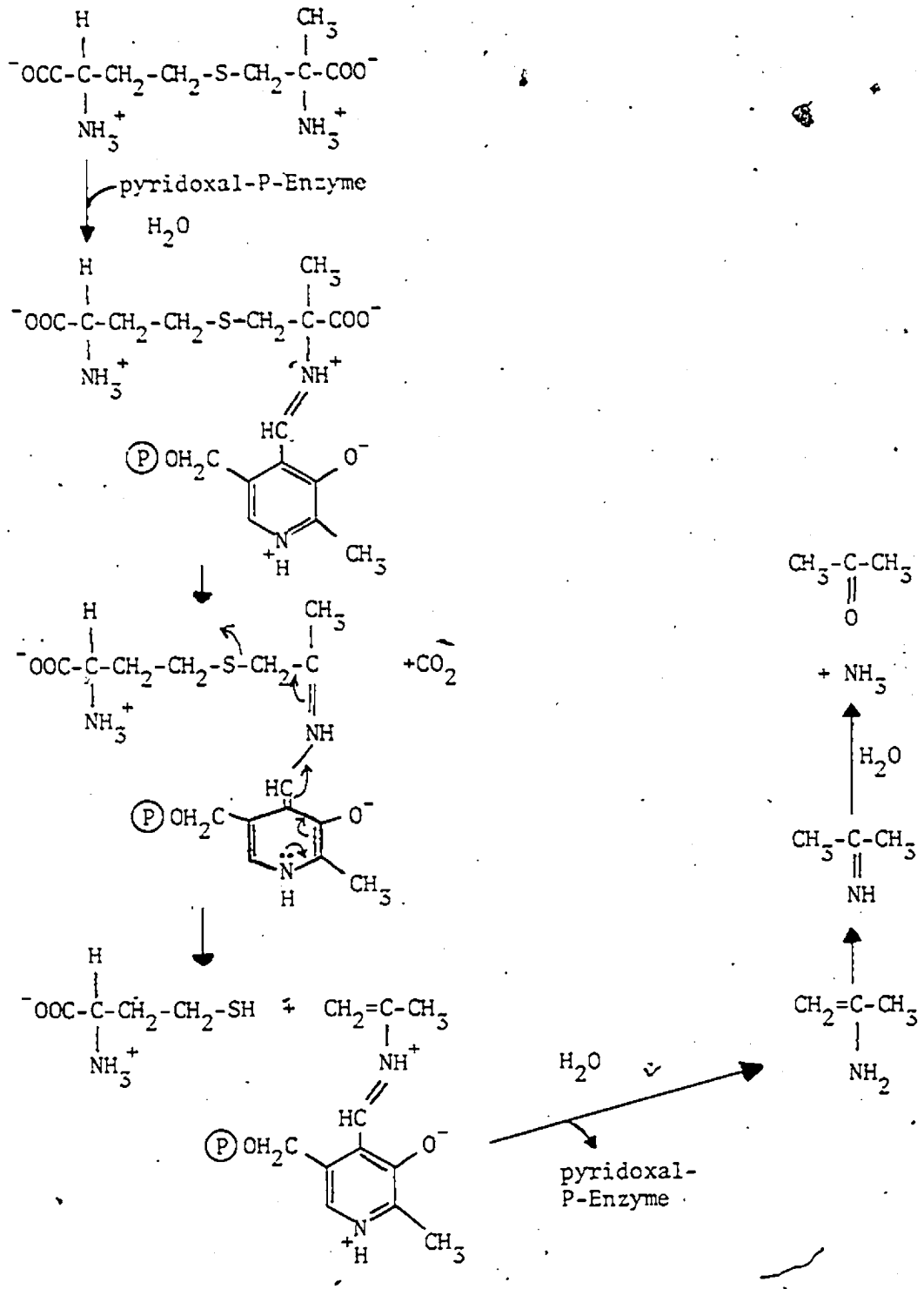
A POSSIBLE REACTION MECHANISM FOR FORMATION OF HOMOCYSTEINE FROM α-METHYL-SUBSTITUTED CYSTATHIONINE

Legend

See text.

FIGURE 12

A POSSIBLE REACTION MECHANISM FOR FORMATION OF HOMOCYSTEINE FROM α-METHYL-SUBSTITUTED CYSTATHIONINE



CHAPTER VI

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PART II

THIN-LAYER CHROMATOGRAPHY OF DANSYL AMINO ACIDS ON POLYAMIDE

CHAPTER I

INTRODUCTION

Introduced by Weber (1) in 1952, 1-dimethylaminonaphthalene-5-sulfonyl chloride has been often used for fluorescence labelling of proteins in order to investigate the physical properties of these macromolecules in solution (2). Weber (1) deduced that the fluorescent dimethylaminonaphthalenesulfonyl residue was attached to the protein primarily through interaction of the sulfonyl chloride with the free amino groups to form sulfonamide linkages. Hartley and Massey's research (3) on the reaction of dansyl chloride with α-chymotrypsin led them to propose its use as a means of studying the α-amino and other reactive groups of peptides and proteins. Gray and Hartley (4-6) were the first to: characterize the reaction of dansyl chloride with amino acids, peptides and proteins; study the properties of dansyl amino acids; and develop experimental techniques for applications of dansyl chloride. Their work has led to the establishment of dansyl chloride as a widely used, multi-purpose analytical reagent in amino acid, peptide and protein chemistry. The development of dansyl chloride methodologies is reviewed here. Unless otherwise indicated, the fundamental details have been taken from the reports of Gray (6-8).

Dansyl chloride is a typical aromatic sulfonyl chloride and, as such, reacts with a variety of nucleophiles. The most important of these are the primary and secondary amines present in free amino acids and as the terminal amino groups of peptides and proteins. Reactive side-chain functions include thiol (cysteine), phenolic hydroxyl (tyrosine), amino (lysine), and imidazole (histidine) in decreasing

order of reactivity. There is essentially no reactivity toward guanidinium (arginine), aliphatic hydroxyl (serine or threonine), amides (asparagine and glutamine), or indole (tryptophan) under normal conditions of pH and reagent concentration.

Amino groups react only as the free base ($R\text{-}NH_2$) and not as the conjugate acid ($R\text{-}NH_3^+$), so labelling must be carried out at alkaline pH. Since at alkaline pH, dansyl chloride is hydrolyzed by OH^- (increasingly dominant above pH 10) as well as water (independent of pH above pH5) to the sulphonic acid (DNS-OH), labelling of most amino acids is optimal in the range of pH 9.5 to 10.5. At lower pH values the unreactive protonated form of the amino acid slows the labelling reaction relative to the hydrolysis by water. At higher pH values the reagent is hydrolyzed too rapidly by hydroxyl ion. In practical labelling experiments it is usual to add a several-fold excess of reagent, and allow the unused dansyl chloride to hydrolyze to DNS-OH. However, successful labelling is not dependent on the ratio of dansyl chloride to reactive groups, but rather on the absolute concentration of dansyl chloride. This is due to the fact that labelling is always in competition with hydrolysis. A minimal dansyl chloride concentration of 5mM has been recommended. To achieve adequate concentrations of DNS-Cl, it is necessary to use a mixed organic-aqueous system (usually 50% acetone (v/v)). The rate of reaction is about 8-fold lower in 50% acetone (v/v) than in water, but the extent of labelling is unaffected. Further optimization of the labelling technique was provided by Gros and Labouesse (9) after a thorough study of the kinetics of labelling and

hydrolysis. Zanetta et al. (10) generally confirmed the work of Gros and Labouesse.

Regarding stability of dansyl amino acids during acid hydrolysis of dansyl peptides and proteins (6 N HCl, 105°, 18 hours), primary sulfonamide derivatives are generally very stable and good recoveries of dansyl amino acids are obtained. DNS-Pro, having a secondary sulfonamide link is more susceptible to acid cleavage, resulting in liberation of DNS-OH. Elimination of $DNS-NH_2$ is the major route of breakdown of a small percentage of DNS-Ser, rather than hydrolysis of the sulfonamide link; the same is likely to be true of DNS-Thr and DNS-Cys. DNS-Trp is usually completely destroyed during acid hydrolysis, resulting in liberation of $DNS-NH_2$, DNS-OH and traces of DNS-Gly, DNS-Ser, and DNS-Ala. Thiol and imidazole derivatives are also unstable during this process, resulting in liberation of DNS-OH and the simple sulfonamide derivative. Gros and Labouesse (9) have reported markedly higher rates of breakdown for derivatives hydrolyzed at 110°. Neadle and Pollitt (11) have examined a side reaction during the labelling reaction whereby a dansyl amino acid can be split into $DNS-NH_2$, carbon monoxide, and the carbonyl compound that has one carbon less than the parent amino acid. The reaction is believed to be dependent on the structure of the amino acid and the reaction conditions (promoted by excess reagent and high pH values) and involve the attack of a second molecule of dansyl chloride on the dansyl amino acid.

After labelling of amino acids, peptides or proteins and acid hydrolysis in the case of peptides and proteins, the various dansyl

amino acids are separated for identification and possibly, quantification. A high-voltage ionophoresis method for separation was developed by Gray and Hartley (4-8). For this purpose an apparatus of the cooled, flat-bed type (12, 13) is necessary, since most dansyl amino acids are extracted into the organic solvents used as coolants in the Michl (14) type of apparatus. Two or three systems are sufficient to identify any of the derivatives of amino acids normally found in protein. Best resolution is obtained by high-voltage ionophoresis (80 v/cm, 2.5-3 h, 15°) on a sheet of Whatman No. 3MM paper at pH 4.40 (0.8% acetic acid - 0.4% pyridine). Boyer and Talalay (15) have reported similar results at pH 4.55. If necessary, remaining uncertainties are cleared up by ionophoresis (20-30 v/cm, 2 h, 15°) at pH 12.7 (0.1 M Na_3PO_4 - 0.1 M NaOH) or by ionophoresis (50 v/cm, 2 h, 20°) at pH 1.9 (8% formic acid). Dansyl peptides have also been separated by ionophoresis (80 v/cm, 1-1.5 hr) at pH 6.5 (0.4% acetic acid - 10% pyridine) on paper (5, 6, 16, 17). Sajgo (18) separated dansyl amino acids by thin-layer electrophoresis on silica gel plates. A system combining adsorption chromatography and high-voltage electrophoresis on cellulose thin-layer plates for the separation of all common dansyl amino acids in a single analysis was proposed by Arnott and Ward (19).

Numerous other separation procedures have been reported. These will be surveyed briefly. Comprehensive reviews have been published (20-22). Several systems for separation by paper chromatography are reported by Hartley and Massey (23) and Boulton and Bush (24) for use when the electrophoretic apparatus is unavailable. One- and two-

dimensional thin-layer chromatography systems for the separation of dansyl amino acids and dansyl peptides have been developed in several laboratories. Shorter separation times and the lack of need for specialized equipment are two advantages over the electrophoretic and paper chromatographic procedures. Also, because of reduced layer thickness, less sample is required and lower detection limits are possible. Thin-layers of silica gel (9, 25-41) have been used most often. Deyl and Rosmus (26) have also used aluminum oxide. A system whereby dansyl amino acids are chromatographed simultaneously on three sorbents (silica gel, alumina oxide and polyamide) using a single solvent has been reported by Deyl and Rosmus (42).

Introduced by Woods and Wang (43) and elaborated by several others (10, 44-49), thin-layer chromatography on polyamide (ε-polycaprolactam) sheets has become the method of choice in many laboratories. A problem with silica gel is the rapid destruction of the derivatives, so that chromatograms have to be examined and recorded immediately. Dansyl derivatives are much more stable on the polyamide layer, and this is a significant advantage of this medium. If desired, the polyamide sheets can be washed in a suitable solvent as soon as possible after development, dried and used again (50).

A method for the separation of water soluble dansyl amino acids on a cation exchange column with hydrophilic crosslinks was reported by Munier and Drapier (51). Nota et al. (52) introduced a liquid-solid chromatography procedure for the separation of dansyl amino acids.

Especially in the case of proteins, resolution in many separation

procedures is adversely affected by excess amounts of salts, dansyl chloride, and DNS-OH. Excess reagent can be hydrolyzed to DNS-OH by acid or alkali (9, 31, 36). Zanetta et al. (10) have precipitated excess dansyl with 0.1 M ammonium formate. In the case of proteins, before hydrolysis, the salts and excess DNS-OH can be removed by Sephadex G-25 column chromatography, dialysis or protein precipitation (7). In the case of peptides and amino acids, DNS-OH has been removed by ion-exchange chromatography using Dowex 50 x 8 (32, 33). Extraction of most dansyl amino acids into ethylacetate saturated with water (7) or ether (9, 31) has been of use, but some dansyl amino acids remain in the aqueous layer and therefore, it must still be examined.

After separation the dansyl amino acids or dansyl peptides can be visualized by irradiation with ultraviolet light. Either long-wave (366 mμ) or short-wave (254 mμ) light is suitable, but the former is preferred. The limits of detection by the eye depends on a number of factors, including, compactness of the spot and thickness of the medium. Also, many substances quench the fluorescence of dansyl amino acids, including water, acids and pyridine. Fluorescent intensity of dansyl derivatives is low in the protonated form and strong in alkaline media (53). Fluorescent intensity is increased after exposure to ammonia fumes (8) or by spraying with a solution of triethanolamine in isopropanol (53). Generally, the limit of detection by eye on paper is between 10^{-11} to 10^{-10} moles of dansyl amino acid and on thin-layer, 10^{-12} to 10^{-11} moles (8). The individual spots are qualitatively identified by comparing their mobility or Rf's to those of dansyl amino acid standards.

Dansyl derivatives can be quantitated in a variety of ways: in situ fluorimetry; fluorimetry after elution; liquid solid-column chromatography with fluorescence recording (52); use of radioactive reactants followed by either autoradiography and subsequent microdensitometry (45) or scintillation counting after elution. Various methods are reported for in situ fluorescence measurements of paper chromatograms and electropherograms (54) and thin-layer chromatograms (20, 55, 55-57). Using polyamide sheets, Varga and Richards (58) developed direct fluorescence scanning techniques with a lower limit of detection at 10^{-14} moles. Alternatively, after a spot is scraped off a thin-layer plate, the dansyl amino acid is extracted from the scrapings with a suitable solvent and estimated by measurement of the fluorescence of the extraction solution (9, 32, 34, 53, 59).

Several workers (60-62) have used [^{3}H] or [^{14}C] dansyl chloride for labelling. After chromatography the spots are scraped off, eluted, and counted. Airhart et al (63) have quantitated dansyl amino acid by fluormetric and radioactive techniques. The problem with these methods is that the yield of the labelling reaction for each amino acid must be estimated by determination of the yields in parallel experiments with amino acid standards. This problem has been overcome by the use of [^{3}H] dansyl chloride with ^{14}C-labelled amino acids as internal standards to monitor the yields of the respective dansyl derivatives (48, 64, 65). The ratio of ^{3}H- to ^{14}C-activities in each dansyl amino acid depends only on the specific activity of the dansyl chloride and on the specific activity of the amino acid reacting with it. The latter is dependent on

the extent of the dilution of the added ^{14}C-labelled amino acid by the amount of the amino acid present in the sample.

Numerous applications of dansyl chloride in amino acid, peptide and protein chemistry have been proposed. The list that follows is by no means complete, but does serve to indicate the broadness of the field. In 1963, Gray and Hartley (4) (also see Gray (6-8)) published a dansyl chloride methodology for qualitative identification of the N-terminal amino acid in peptides and proteins. The terminal amino acid is derivatized with dansyl chloride. After hydrolysis, the dansyl amino is electrophoresed and qualitatively identified. This method was proposed to be the natural successor to Sanger's 1-fluoro-2,4-dinitrobenzene procedure (66), for a variety of reasons. Owing to the strong fluorescence of the dansyl amino acids, the method is approximately 100 times as sensitive. Consequently, the method is useful when only small amounts of material are available. The method was readily applicable to 10^{-10} to 10^{-9} moles of material. This sensitivity has made it possible to use extremely simple handling methods, making the procedure more rapid and reliable. Also, the dansyl amino acids are more stable to acid hydrolysis than the corresponding dinitrophenyl-amino acids. Of course, dansyl amino acids can be separated by any of the other methods and even quantitated if desired. Gros and Labouesse (9) determined the N-terminal amino acids of several enzymes using a quantitative procedure requiring only between 5×10^{-9} and 10^{-8} moles of enzyme.

This principle has been extended for peptide "mapping". After

cleavage of the protein, the various peptides are reacted with dansyl chloride. The dansyl peptides are separated, eluted and the N-terminal amino acid determined in the usual manner. Atherton and Thomson used the technique to compare the ATP: creatine phosphotransferases from bovine brain, bovine muscle and rabbit muscle. Zanetta et al. (10) modified solvent systems to resolve, for example, 61 spots from a tryptic hydrolyzate of bovine pancreatic RNAase by silica gel thin-layer chromatography; dansyl peptides could be detected at 10^{-11} mole levels, and a reliable "map" obtained with 10^{-10} to 10^{-9} moles. Up to 20 nmoles per silica gel plate could be separated, providing a fast method for isolation of peptides for structure work. Properly handled, 1.5×10^{-8} to 3.5×10^{-8} moles provided N-terminal amino acid determination, amino acid composition, tryptic fingerprinting, and analysis of the peptides. On a ultramicro scale, Spivak et al. (40), made quantitative estimations on 10^{-11} to 10^{-10} moles of insulin β chain hydrolyzed by several enzymes.

Also, in 1963, Gray and Hartley (5) (also see Gray (16)) and Hood, Gray and Dreyer (17), reported a procedure whereby the dansyl N-terminal amino acid method is coupled with Edman phenyl isothiocyanate reaction (67) for the stepwise chemical procedure for the degradation of peptides to produce a sensitive method for peptide sequencing. A sample of peptide is subjected to successive cycles of phenyl isothiocyanate degradation. At the end of each cycle an aliquot of peptide, containing one less amino acid than the previous peptide sample, is removed. The N-terminal amino acid of each peptide is determined as in peptide "mapping", thus revealing the amino acid sequence. Also, by

interpretation of the electrophoretic mobilities of dansyl peptides (based on Offord's system (68) derived for free peptides), the position and number of amide groups in peptides can be determined. Approximately 10^{-9} to 5×10^{-9} moles was required for each degradation step, i.e., 2×10^{-8} moles was usually sufficient to establish the complete sequence of a penta- or hexapeptide. With slightly more material, amide groups could also be determined. Pataki (69) introduced the double-checking technique, whereby the N-terminal acid for a peptide by dansylation is checked against the phenylthiohydantoin derivative released during the subsequent degradation cycle. For increased sensitivity, Fambrough and Bonner (70) have reported a method for the conversion of phenylthiohydantoin derivatives to dansyl derivatives. Ultramicro techniques have been reported (40, 71). Gray and Smith (72) reported a rapid method for small peptides in which 0, 1, 2, 3, 4 cycles of Edman degradation are performed with extraction respectively on 5 aliquots of a peptide, followed by labelling of each portion to determine the N-terminal residue. Percy and Buchwald (73) determined 15 to 20 residues of human immunoglobin light chains.

Dansyl chloride methodologies have been used for the estimation of free amino acids in biological fluids and tissues. Superfusates of the cerebral cortex in rats was examined by Crowshaw et al. (34) and Snodgrass and Iversen (64). Briel et al. (60) have studied substances in the spinal cord of the cat. Free amino acids in the rat optic nerve have been investigated by Casola and DiMatteo (61). Joseph and Halliday (65) have easily determined the most abundant amino acids in rat brain

tissue (1 mg).

A variety of miscellaneous uses include: dansylation as an alternative to conventional staining techniques of proteins for gel electrophoresis (74, 75); methods to monitor solid phase Merrifield peptide synthesis and solid phase Edman degradation (76); determination of catecholamine metabolites (78).

It has been mentioned earlier that thin-layer chromatography on polyamide layers (10, 43, 45-49) has become the method of choice in many laboratories for the separation of dansyl amino acids. After two-dimensional chromatography in those systems in which water-formic acid (100: 1.5-3 v/v) (solvent 1) and benzene-acetic acid (9:1 v/v) (solvent 2) are used as the first two solvents (43, 45-48) several dansyl amino acids remain unresolved. The problems include: the pairs DNS-Asp/DNS-Glu, DNS-Thr/DNS-Ser and DNS-NH_2/DNS-Ala; the quartet DNS-Arg/DNS-His/α-DNS-Lys/ε-DNS-Lys; and the obscuring of DNS-CysSO_3H in the presence of the usual excessive amounts of DNS-OH. Also, when DNS-Gln and DNS-Asn are present, they run together with DNS-Thr and DNS-Ser resulting in an unresolved quartet (60). In the system of Lee and Safille (49), (solvent 1, water-formic acid (100:1.5 v/v); solvent 2, benzene-acetic acid (4.5:1 v/v)), fewer dansyl amino acids remain unresolved. They include the pairs DNS-NH_2/DNS-Ala and DNS-Cys SO_3H/DNS-OH, the triplet DNS-Arg/α-DNS-Lys/ε-DNS-Lys, and possibly the quartet DNS-Gln/DNS-Thr/DNS-Asn/DNS-Ser.

The resolution of these dansyl amino acids has only been achieved after further chromatography. Hartley (46) utilized ethyl

acetate-methanol-acetic acid (20:1:1 v/v) (Crowshaw et al (34)) as a third solvent in the solvent 2 direction. Ambiguities between ε-DNS-Lys, DNS-His and DNS-Arg that still remain were resolved by a fourth solvent, 0.05 M trisodium phosphate-ethanol (3:1 v/v) in the solvent 2 direction. DNS-$CysSO_3H$ which is still obscured by DNS-OH, can be resolved by chromatography with a fifth solvent, 1 M ammonia-ethanol (1:1 v/v) in the solvent 2 direction. Alternatively, DNS-OH could have been removed before thin layer chromatography by ion exchange chromatography (32, 33). For a third solvent in the solvent 2 direction, Lee and Safille (49) used either the third solvent of Hartley (46) or a solvent consisting of two parts ethyl acetate to 10 parts of benzene-acetic acid (4.5:1 v/v). Presumably, solvents four and five of Hartley (46) would be required for resolution of all the dansyl derivatives of amino acids normally encountered in proteins. Brown and Perham (48) used n-butyl acetate-methanol-acetic acid (30:20:1 v/v) as the third solvent. The thin-layer sheet was developed twice with this solvent in the solvent 2 direction. No further problems were encountered in their system at this point. However, the dansyl derivatives of lysine were not present to interfere with the resolution of DNS-Arg and DNS-His; and DNS-$CysSO_3H$ which would have been obscured by DNS-OH was also omitted.

In the polyamide thin-layer chromatography procedures outlined above, one of the problems is the obscuring of DNS-$CysSO_3H$ by DNS-OH. This dilemma can be solved by an additional chromatography step or by removal of DNS-OH. From the chromatographic procedure in PART I, it is

observed that DNS-CysSO_3H migrates ahead of CysSO_3H on polyamide when water-pyridine-acetic acid-formic acid (93:4.5:0.8:1.0 v.v) was used as a solvent. Thoughts concerning the applicability of this solvent as a replacement for the conventional water-formic acid (100:1.5-3 v/v) solvent provided the impetus for this project. It was hoped that this solvent (or a slightly modified version) in combination with benzene-acetic acid (4.5:1 v/v) (49) would alone result in the separation on polyamide layers of all the dansyl derivatives of the amino acids commonly found in proteins.

CHAPTER II

A. MATERIALS

Polyamide sheets (Macherey-Nagel Polygram Polyamide-6) were obtained from Brinkmann Instruments, Toronto, Ontario. Amino acids commonly found in proteins and cysteic acid, methionine sulfoxide and methionine sulfone were purchased from Sigma Chemical Co., St. Louis, MO. Dansyl chloride and ε-DNS-Lys were obtained from Pierce Chemical Company, Rockford, Il. All other chemicals and solvents used were analytical grade.

B. METHODS

Dansyl amino acids were prepared in a manner similar to that of Gros and Labouesse (9). Approximately 1 mg of each amino acid was dissolved in 0.5 ml of 0.5 M $NaHCO_3$. A 0.2-ml aliquot of the amino acid solution was transferred to a small test tube. After addition of a 0.2-ml aliquot of a dansyl chloride solution (5 mg/ml acetone), the tube was sealed with parafilm and incubated at 37° for 1 h. The reaction was stopped by addition of 0.6-ml of 10% (v/v) formic acid. Appropriate changes were made when a mixture of dansyl amino acids was prepared. The dansyl amino acids were not isolated and were used, as prepared, as reference compounds in chromatography.

Chromatography was performed using the Eastman Chromogram Developing Apparatus 6071 (Eastman Kodak Co., Rochester, N.Y.). The relative positions of the dansyl amino acids after one-dimensional chromatography was determined using each of the following solvents:

(a) water-formic acid (100:3 v/v) (43)

(b) water-pyridine-acetic acid-formic acid (83:4.5:0.8: 1.0 v/v)

(c) water-pyridine-formic acid (93:4.5:2.5 v/v)

(d) water-pyridine-formic acid (93:3.5:3.5 v/v)

(e) benzene-acetic acid (9:1 v/v) (43)

(f) benzene-acetic acid (4.5:1 v/v) (49)

Leaving 3 cm from each edge, eight dansyl amino acids were individually spotted 2 cm apart in a line 3 cm from the bottom of a 20 x 20 cm polyamide sheet. Chromatography was stopped when the solvent front had moved about 15 cm. The dansyl amino acids were visualized by long wave ultraviolet radiation (UVL 56, Ultra-violet Products Inc., San Gabriel, CA).

Two-dimensional chromatography using the following solvents systems:

(a) water-formic acid (100:3 v/v) followed by benzene-acetic acid (9:1 v/v) (43)

(b) water-formic acid (100:3 v/v) followed by benzene-acetic acid (4.5:1 v/v) (49)

(c) water-pyridine-acetic acid-formic acid (93: 4.5:0.8: 1.0 v/v) followed by benzene-acetic acid (4.5:1 v/v)

(d) water-pyridine-formic acid (93:4.5:2.5) followed by benzene-acetic (4.5:1 v/v)

(e) water-pyridine-formic acid (93:4.5:2.5) followed by benzene-acetic (4.5:1 v/v),

was performed to assess the ability of these systems to separate the dansyl amino acids contained in a mixture. The dansyl amino acids were spotted in a corner approximately 3.0 cm from each edge of a 20 x 20 cm

polyamide sheet. Development in both directions was stopped when the solvent front had moved about 15 cm. The chromatogram was dried in a stream of cool air before development in the second dimension.

CHAPTER III

RESULTS AND DISCUSSION

Diagrammatic representations of chromatograms obtained after two-dimensional chromatography using water-formic (100:3 v/v) in the first direction and benzene-acetic acid, either 9:1 v/v (43) or 4.5:1 v/v (49) in the second direction, are shown in Figures 1 and 2, respectively. Those problems of resolution for each of these systems described in the INTRODUCTION were generally confirmed. Furthermore, after chromatography using the system of Lee and Safille (49), results in our laboratory showed that DNS-MetSO was in a position similar to that which these workers designated as DNS-$MetSO_2$ and that DNS-$MetSO_2$ partially overlapped with DNS-Thr. It is also interesting to point out the difference in mobility between α-DNS-Lys and ε-DNS-Lys in benzene-acetic acid (4.5:1 v/v) which had previously been undetected (49). Three spots, one obviously corresponding to bis-DNS-Lys, were observed when lysine was derivatized and chromatographed in one-dimension using this solvent. The other two spots designated α-DNS-Lys and ε-DNS-Lys in this report were confirmed by chromatographic comparison with the standard ε-DNS-lys purchased from Pierce and on the basis of the observation that both migrated together when chromatographed using water-formic acid (100:3 v/v) (43, 49).

A diagrammatic representation of the chromatogram obtained with the solvent system comprised of water-pyridine-acetic acid-formic acid (93:4.5:0.8:1.0 v/v) and benzene-acetic acid (4.5:1 v/v) is shown in Figure 3. This solvent system which had provided the initial stimulus for

FIGURE 1

CHROMATOGRAPHY OF DANSYL AMINO ACIDS

Legend

Diagrammatic two dimensional separation of the common dansyl amino acids on polyamide. Chromatographic solvents: Direction 1, water-formic acid (100:3 v/v); Direction 2, benzene-acetic acid (9:1 v/v).

FIGURE 1

CHROMATOGRAPHY OF DANSYL AMINO ACIDS

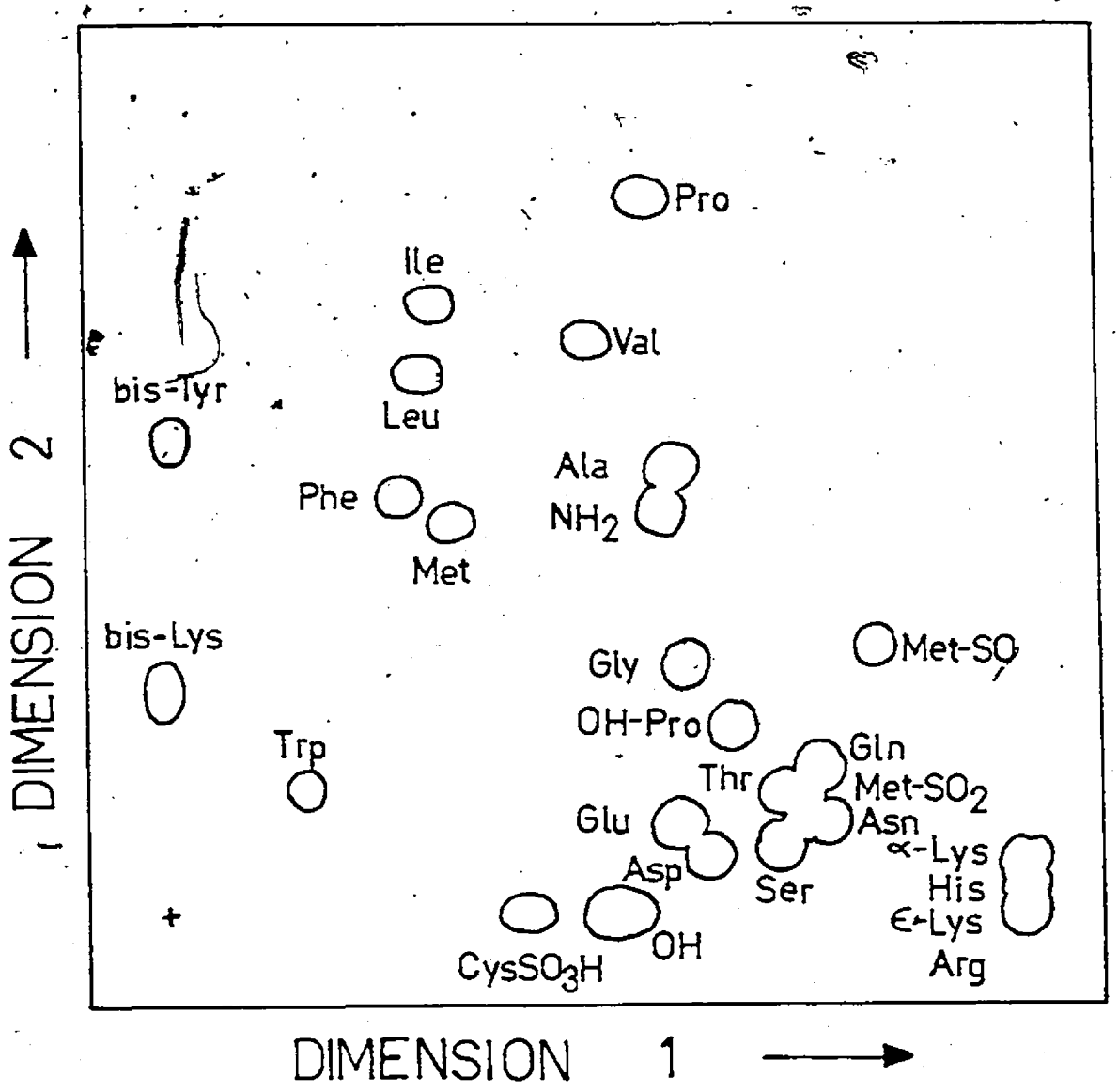


FIGURE 2

CHROMATOGRAPHY OF DANSYL AMINO ACIDS

Legend

Diagrammatic two-dimensional separation of the common dansyl amino acids on polyamide. Chromatographic solvents: Direction 1, water-formic acid (100:3 v/v); Direction 2, benzene-acetic acid (4.5:1 v/v).

FIGURE 2

CHROMATOGRAPHY OF DANSYL AMINO ACIDS

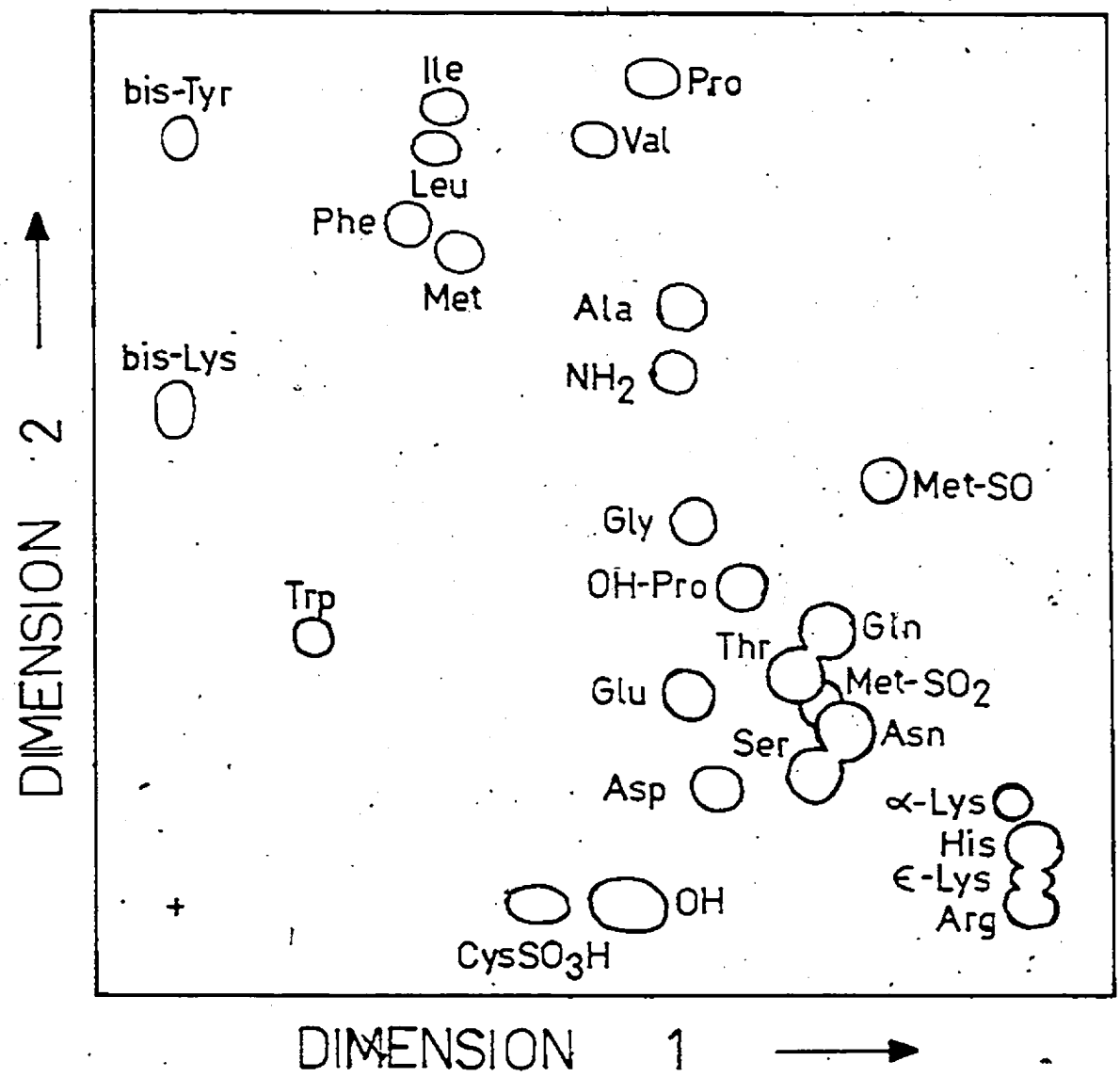


FIGURE 3

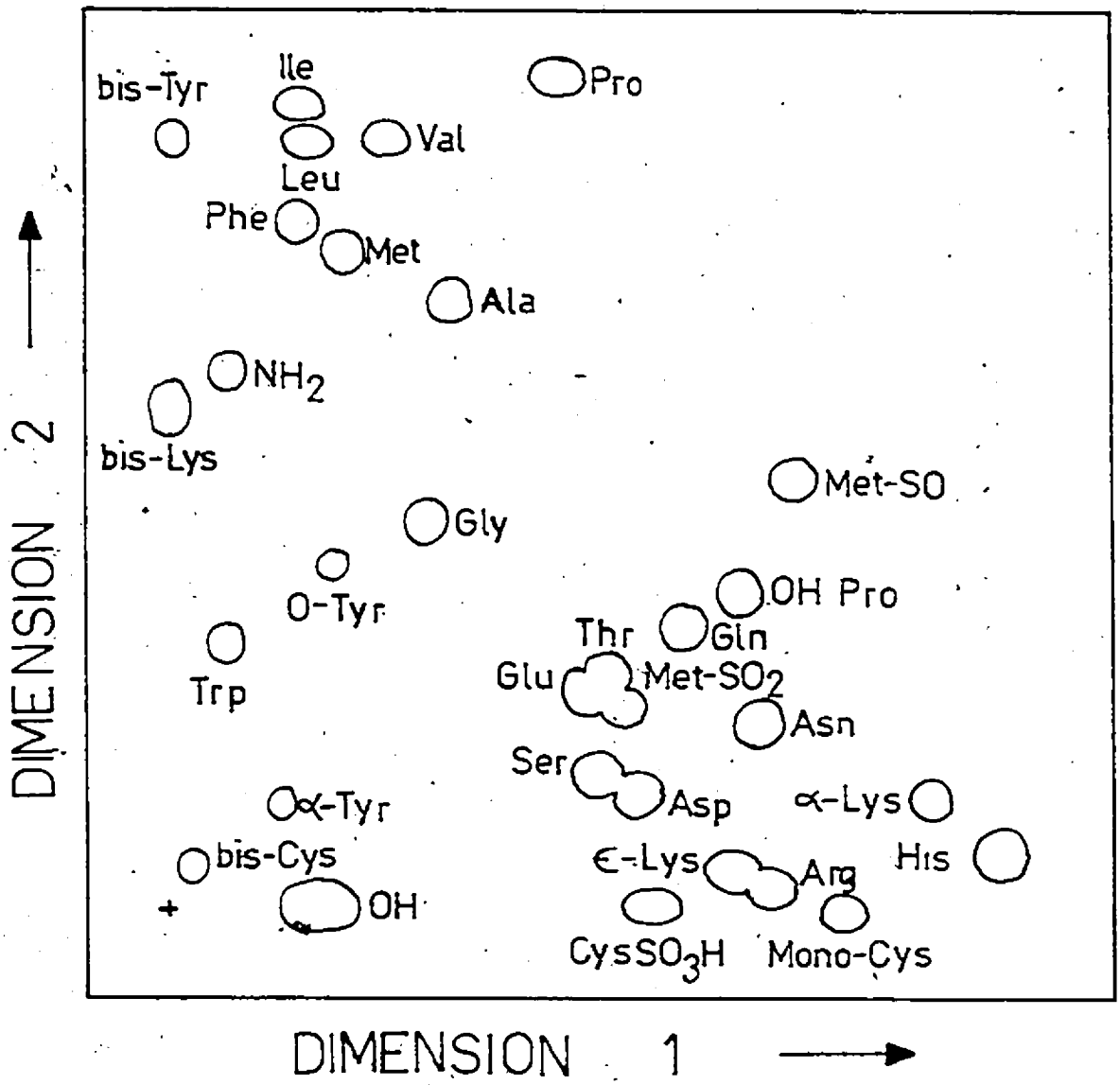
CHROMATOGRAPHY OF DANSYL AMINO ACIDS

Legend

Diagrammatic two-dimensional separation of the common dansyl amino acids on polyamide. Chromatographic solvents: Direction 1, water-pyridine-acetic acid-formic acid (93:4.5:0.8:1.0 v/v); Direction 2, benzene-acetic acid (4.5:1 v/v).

FIGURE 3

CHROMATOGRAPHY OF DANSYL AMINO ACIDS



this work proved helpful in several ways and detrimental in others. As expected DNS-$CysSO_3H$ was easily separated from DNS-OH, $DNS-NH_2$ and DNS-Ala were now well separated. DNS-Gln and DNS-Asn were resolved from each other and now quite removed from DNS-Thr and DNS-Ser. Some advancements were made in the resolution of the quartet DNS-Arg/DNS-His/α-DNS-Lys/ε-DNS-Lys which was a major problem in the previous systems. Only DNS-Arg and ε-DNS-Lys were not completely resolved. The drawback to the system was that it introduced two not completely resolved pairs, i.e., DNS-Thr/DNS-Glu and DNS-Ser/DNS-Asp. The presence of $DNS-Met-SO_2$ resulted in further complications.

A diagrammatic representation of the chromatogram obtained when water-pyridine-formic acid (93:4.5:2.5 v/v) was used in the first direction is shown in Figure 4. In addition to the previous improvements, the pairs DNS-Thr/DNS-Glu amd DNS-Ser/DNS-Asp were now resolved. The pairs $DNS-Thr/DNS-MetSO_2$ and DNS-Arg/ε-DNS-Lys were still not totally resolved. DNS-Gln and DNS-OH-Pro overlapped slightly, but otherwise no new problems were present.

The solvent comprised of water-pyridine-formic acid (93:4.5:2.5 v/v) was modified slightly in several ways (more or less pyridine with more or less formic acid). The chromatogram obtained when water-pyridine-formic acid (93:3.5:3.5 v/v) was used in the first direction provided optimal resolution and diagrammatic representation is shown in Figure 5. The problems with DNS-Arg/ε-DNS-Lys and DNS-Gln/DNS-OH-Pro were solved with no new problem resulting. DNS-Thr and $DNS-MetSO_2$ continued to partially overlap. The degree of separation was suitable for

FIGURE 4

CHROMATOGRAPHY OF DANSYL AMINO ACIDS

Legend

Diagrammatic two-dimensional separation of the common dansyl amino acids on polyamide. Chromatographic solvents: Direction 1, water-pyridine-formic acid (93:4.5:2.5 v/v); Direction 2, benzene-acetic acid (4.5:1 v/v).

FIGURE 4

CHROMATOGRAPHY OF DANSYL AMINO ACIDS

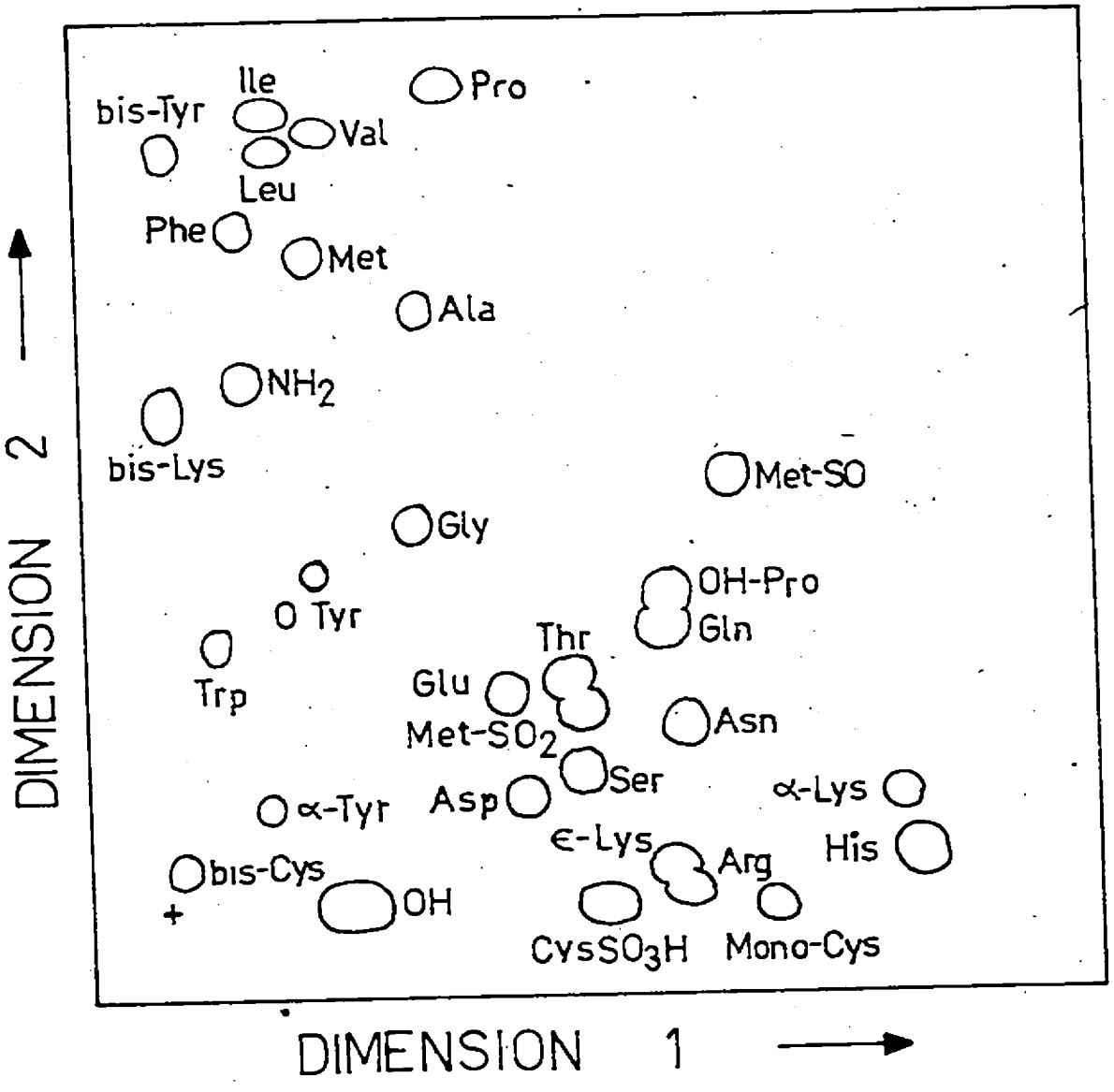


FIGURE 5

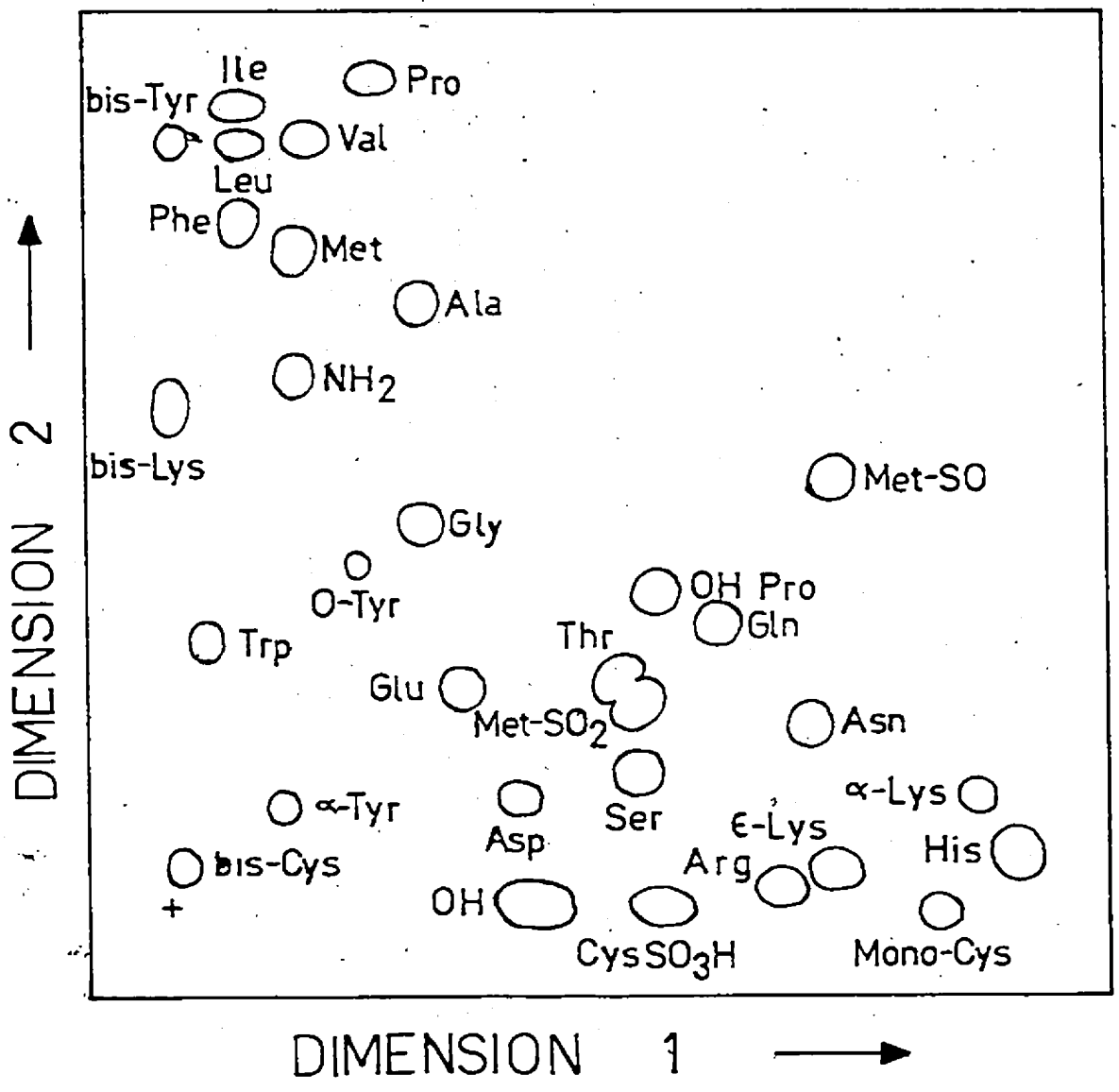
CHROMATOGRAPHY OF DANSYL AMINO ACIDS

Legend

Diagrammatic two-dimensional separation of the common dansyl amino acids on polyamide. Chromatographic solvents: Direction 1, water-pyridine-formic acid (93:3.5:3.5 v/v); Direction 2, benzene-acetic acid (4.5:1 v/v).

FIGURE 5

CHROMATOGRAPHY OF DANSYL AMINO ACIDS



qualitative identification. For quantitation, the two can be separated by repeating the chromatography in the second direction with the same solvent, benzene-acetic acid (4.5-1 v/v).

CHAPTER IV

SUMMARY AND CONCLUSIONS

The development of a two-dimensional thin-layer chromatography system for the separation of the dansyl derivatives of the amino acids commonly found in proteins has been described. Of those solvent systems tested, chromatography using water-pyridine-formic acid (93:3.5:3.5 v/v) in the first direction and benzene-acetic acid (4.5:1 v/v) in the second direction provided optimal separation. The previously developed systems would require either five chromatographic solvents or four chromatographic solvents, preceded by the removal of DNS-OH from the reaction mixture, to even approximate the degree of separation achieved with this new system.

CHAPTER V

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PART III

A COMPETITIVE PROTEIN BINDING ASSAY FOR INDIVIDUAL PLASMA ESTROGENS

CHAPTER I

INTRODUCTION

In non-pregnant women the two primary estrogens are 17β-estradiol and estrone. Synthesis of these steroids occurs mainly in the ovaries (1) with small amounts being produced in the adrenal cortex. Minor amounts of 17β-estradiol and estrone are synthesized in the adrenal cortex and testes of males. While both 17β-estradiol and estrone are secreted directly from the ovary, a majority of the circulating estrone arises from the metabolism of circulating C_{19} neutral steroids, e.g. androstenedione (2). Estrone and 17β-estradiol are interconvertible (3). Estriol is a metabolic product of 17β-estradiol and estrone (4). The most potent of these three estrogens is 17β-estradiol, followed by estrone and then estriol. Estrogens initiate and maintain maturity of the female genitalia and the secondary sex characteristics. Together with progesterone, they maintain and control menstruation. Consequently assay of plasma 17β-estradiol and estrone may be useful in the evaluation of menstrual cycle difficulties due to hypogonadotropic and hypoestrogenic conditions, sexual maturity in females, feminization in children, and in the detection of estrogen-producing tumors. In fertility studies, estradiol measurements may be of help to establish the time of ovulation and optimal time for conception.

In pregnancy, the major source of estrogens is the placenta. Estriol, synthesized from precursors of primarily fetal origin, is the major estrogen produced. In normal pregnancies the levels of estriol in blood and urine continue to rise throughout the third trimester of pregnancy. Consistently low estriol levels or a sudden drop observed in

serial determinations is indicative of fetal-placental distress (5, 6).

The circulating estrogens are present in the form of free, conjugated, and protein-bound steroid. Most of the plasma methods for the assay of the estrogens do not include hydrolysis and, therefore, measure free and protein-bound estrogens. Normal plasma values in non-pregnant females for 17β-estradiol, estrone and estriol are in the ranges 20-300 pg/ml, 40-200 pg/ml and less than 200 pg/ml, respectively. The range for plasma estriol during the third trimester of pregnancy is 3-20 ng/ml. Methods for their determination must possess a high level of sensitivity. Methods for plasma estrogens have been reported using gas chromatography (7-9) and double isotope derivative techniques (10). Although these methods are sufficiently sensitive, they require large volumes of plasma which preclude their use in studies where frequent samples from the same patient are required. The methods are also relatively complex.

Radioimmunoassay has become the most popular method within the last several years (11-16). Many commercial estrogen radioimmumoassay kits are presently marketed. Most antibody preparations exhibit significant cross-reactive properties and so the estrogens must be separated prior to assay. Present research in this area is directed towards the preparation of antibodies specific to a single estrogen. Such preparations would allow assay using untreated plasma samples.

Methods for competitive protein binding assay of plasma estrogens have been reported (17-20). The methodology is similar to radioimmunoassay except that the high-affinity estrogen-binding protein present

in the cytosol of uterine tissue replaces the antibody. Estrogen-binding proteins first discovered in 1962 by Jensen and Jacobson (21), have received a great deal of attention and several reviews have been published (22-24). Competitive protein-binding assay is more sensitive than radioimmunoassay, c.f. 4 pg (18) versus 50 pg (12). However, the method has not been widely used. Since the estrogen receptors are very unstable, commercial distribution has not been possible. Probably, a comparatively small number of hospital laboratories have the necessary facilities and equipment, or the desire, to prepare the uterine cytosol.

The purpose of this study was to "develop" a competitive protein binding assay for estrogens in the plasma of non-pregnant females for routine use in a local hospital. The hospital laboratory had found that several commercial radioimmunoassay kits, marketed for this purpose, were not sufficiently sensitive.

The various procedures and techniques used for the assay have been adapted from published methods. The procedure for the extraction and separation of plasma estrogens was adapted from that described in the package insert of the Estrogens (E_1/E_2) [^{3}H] Radioimmunoassay Reagent Pak marketed by New England Nuclear (Lachine, Quebec, H7T 3C9). The preparation of calf uterine cytosol was based on that suggested by Jungblut et al. (25). Human uterine cytosol was prepared according to the method of Henderson and Schlach (20). Competitive protein-binding assay was performed primarily as previously described (17-20).

CHAPTER II

EXPERIMENTAL

A. MATERIALS

2, 4, 6, 7 - ^{3}H - Estrone (250 µCi/250 µl benzene-ethanol (9:1 v/v), S.A. 115 Ci/mmol), 2, 4, 6, 7, 16, 17 - ^{3}H-17β-estradiol (250 µCi/250 µl benzene-ethanol (9:1 v/v), S.A. 152 Ci/mmol), 2, 4, 6, 7 - ^{3}H - estriol (250 µCi/250 µl benzene-ethanol (9:1 v/v), S.A. 85 Ci/mmol) and RIAFLUOR scintillator were purchased from New England Nuclear (Lachine, Quebec, H7T 3C9).

Estrone, 17β-estradiol, estriol, dextran (clinical grade, average M.W. 83,300), dithioerythritol and bovine serum albumin were obtained from Sigma Chemical Company (Saint Louis, MO , 63178.)

Sephadex LH-20 was obtained from Pharmacia (Canada) Ltd., (Dorval, Quebec, H9P 1H6).

Charcoal, Norit A (neutral) was purchased from BDH Chemicals (Toronto, Ontario M8Z 1K5).

Biuret reagent and A.C.S. grade diethyl ether were obtained from Fisher Scientific Co. Limited (Don Mills, Ontario, M3A 1A9).

Spectrophometric grade isooctane (2,2,4-trimethylpentane) was purchased from Aldrich Chemical Company (Milwaukee, WI , 53233)

All other chemicals and organic solvents used were analytical grade.

Disposable glass tubes, 12 x 75 mm, were purchased from Fisher Scientific Co. Limited.

Liquid scintillation counting was performed using a Nuclear-Chicago Corp. (Chicago, IL) Unilux II liquid Scintillation System ad-

justed for balance point or high E^2/B counting. Counting efficiency ranged between 22-35% depending on the nature of the sample.

Two outdated pints of whole blood (anticoagulant citrate phosphate dextrose solution) were obtained from Grace Hospital (Windsor, Ontario).

B. REAGENTS

Unless otherwise stated, all reagents were stored at 4°.

1. Buffer Solutions

Tris-HCl buffer (0.01 M, pH 7.5) containing 0.001 M EDTA, 0.001 M DTE and 0.001 M NaN_3 was used in the preparation of the calf uterine cytosols and all other buffer-containing reagents used in experiments with these cytosols. Tris-HCl buffer (0.01 M, pH 8.0) containing 0.25 M sucrose, 0.001 M EDTA and 0.001 M DTE was used in the preparation of human uterine cytosol and all associated buffer-containing reagents.

2. ^{3}H-Estrogen Solutions

Stock solutions of ^{3}H-E_1, ^{3}H-E_2 and ^{3}H-E_3 were prepared by evaporating to dryness a 30-µl aliquot of the appropriate manufacturer's solution and redissolving in 10 ml of absolute ethanol.

Solutions of ^{3}H-E_1, ^{3}H-E_2, ^{3}H-E_3, each containing approximately 1,000 cpm per 50 µl (mass 3.5, 2.7 and 5.1 pg, respectively), were prepared by diluting 1 ml of the appropriate stock solution to 100 ml with absolute ethanol. A solution containing approximately 1,000 cpm of each tritiated estrogen per 50 µl, was prepared by combining 1 ml of each stock solution and diluting to 100 ml with absolute ethanol.

Solutions of ^{3}H-E_1, containing approximately 10,000 cpm (mass 53 pg) and 14,000 cpm (mass 71 pg) per 200 µl, were prepared by evaporating to dryness 0.75 and 1.0 ml-aliquots of ^{3}H-E_1 stock solution, respectively, and redissolving in 20 ml of appropriate buffer. A solution of ^{3}H-E_2 containing approximately 16,000 cpm (mass 54 pg) per 200 µl, was prepared in the same manner from 1.0 ml of ^{3}H-E_2 stock solution. Solutions of ^{3}H-E_3, containing approximately 9,500 cpm (mass 61 pg) and 16,000 cpm (mass 102 pg) per 200 µl were prepared in the same manner from 0.6 and 1.0 ml-aliquots of ^{3}H-E_3 stock solution, respectively.

3. Estrogen Solutions

Solutions of E_1, E_2, and E_3 containing 5,000 pg/ml and 500 pg/ml were prepared in absolute ethanol and appropriate buffer. Solutions containing 5,000 and 500 pg of each estrogen per ml were prepared in absolute ethanol and appropriate buffer.

4. Dextran-Coated Charcoal

Charcoal, Norit A (neutral) was suspended in 1 N HCl (1 g of charcoal / 100 ml of acid solution). After several hours, the suspension was centrifuged at 9,000 x g. The charcoal pellet was suspended in deionized distilled water (1 g / 100 ml) and then centrifuged as before. After three further water rinses, the charcoal was spread on a watch glass and dried in an oven at 60°.

Dextran-coated charcoal was prepared by dissolving 25 mg of dextran in 100 ml of the appropriate buffer and then adding 250 mg of the acid-washed charcoal.

5. Plasma

The whole blood was centrifuged at 13,000 x g for 20 min. The plasma was stored in 15-ml aliquots at -20°.

C. METHODS

1. Extraction of Plasma Estrogens

A 2-ml aliquot of plasma was added to a 14-ml screw cap culture tube. Depending on the nature of the experiment, a known amount of unlabelled estrogen(s) (0-1,000 pg) in a comparatively small volume of appropriate buffer (0-200 µl) was added directly to the plasma. A 50-µl aliquot of absolute ethanol containing approximately 1,000 cpm of the tritiated form of each estrogen to be assayed was also added directly to the plasma sample in order to determine the percent recovery. After mixing, 5 ml of diethyl ether was added. The sample was vortexed for 60 sec and then centrifuged at 2,000 x g for 5 min. The lower portion of the tube was immersed in a dry ice-acetone bath to snap freeze the aqueous layer. The ether layer was decanted into a 21 x 50 mm glass vial and evaporated to dryness under a gentle stream of nitrogen at 45°.

During the extraction recovery study, the ether layer was decanted into a scintillation vial and evaporated to dryness. The residue was dissolved in 10 ml of scintillator and counted.

2. Separation of the Estrogens by Column Chromatography

Separation of estrogens was achieved by column chromatography on Sephadex LH-20. The chromatographic solvent contained isooctane-benzene-methanol (62:20:18 v/v).

A 1 x 15 cm glass column was rinsed with solvent. The column outlet was sealed leaving several ml of solvent and no air in the bottom of the column. A polypropylene grid was placed at the bottom of the column. A filter paper disc 1.07 cm in diameter was positioned on top of the grid.

A slurry of either 800 or 900 mg of Sephadex in approximately 6 ml of the solvent was prepared in a 10-ml beaker. The slurry was transferred to the column and the column outlet was opened. Small amounts of packing material left in the beaker were transferred to the column with several solvent washes. When approximately 10 ml of solvent had passed through the column, a second filter paper disc was placed on the top of the settled bed. The top of the column and column outlet were sealed until use.

The elution patterns of E_1, E_2 and E_3 were determined for both the 800 and 900 mg columns. A 50-µl aliquot of absolute ethanol containing approximately 1,000 cpm of the tritiated form of each estrogen to be monitored was placed into the bottom of a 15 x 45 mm glass vial. In some experiments, a known amount of unlabelled estrogen(s) (0-1,000 pg) in a small volume of absolute ethanol (0-200 µl) was also added to the vial. The sample was evaporated to dryness under a gentle stream of nitrogen at room temperature. The residue was redissolved in 200 µl of chromatographic solvent, applied to the top of the gel bed and allowed to enter into the bed. Another 200 µl of solvent was used to rinse the glass vial. This solvent was applied to the top of the gel bed and allowed to enter into the bed. Known volumes of chromato-

graphic solvents were added and collected in scintillation vials. The eluates were evaporated to dryness under a gentle stream of nitrogen at 37° redissolved in 10 ml of scintillator and counted. The elution patterns were established by repeating the chromatography using various volumes of chromatographic solvent.

Dried ether extracts were prepared for column chromatography in the following manner. The residue in the 21 x 50 mm glass vial was transferred into the bottom of a 15 x 45 mm glass vial using four individual 200-µl aliquots of chromatographic solvent. The combined solution was evaporated to dryness under a stream of nitrogen at room temperature. The residue was transferred to the column as previously described. A 900 mg column was used for the chromatography of all ether extracts. The elution scheme employed was as follows: 4.6 ml, eluate discarded; 2.5 ml, eluate collected (E_1); 0.3 ml, eluate discarded; 4.0 ml, eluate collected (E_2); 3.6 ml, eluate discarded; 7.0 ml, eluate collected (E_3). The eluates were collected in 14-ml screw cap culture tubes. Two methods for the preparation of eluates for recovery determination and assay were investigated. In Method A, an aliquot representing approximately 20% of the eluate was added to a scintillation vial, evaporated to dryness under a gentle stream of nitrogen at 45°, redissolved in 10 ml of scintillator and counted. The remainder was aliquoted into two 12 x 75 mm disposable glass tubes (approximately 40% per tube), evaporated to dryness and saved for assay. In Method B, the eluate was evaporated to dryness and redissolved in 2.0 ml of ethanol. A 0.4-ml aliquot was added to a scintillation vial

containing 10 ml of scintillator and counted. The remainder was aliquoted into two 12 x 75 mm disposable glass tubes (approximately 40% per tube), evaporated to dryness and saved for assay.

3. Preparation of Uterine Cytosols

Calf uteri were obtained through the courtesy of a local abbatoir. The uteri of two freshly killed 14-week old calves were removed and transported to the laboratory in ice-cold buffer. The cytosol from each uterus was prepared as follows. The uterus was weighed and then cut into small pieces approximately 5 x 5 x 2 mm in buffer at 4°. The tissue was homogenized in a Waring blender at 4° in three volumes of buffer using six homogenization periods of 20 sec, each followed by a 60 sec cooling period. The homogenate was centrifuged at 5,000 x g at 2° for 20 min. The supernatant was centrifuged at 100,000 x g for 90 min at 1°. The resulting supernatant, uterine cytosol, was carefully removed and the total volume was measured. Aliquots of both uterine cytosols were removed for protein determination and competitive-protein binding assay. The remainder was stored in 3-ml aliquots at -20°.

A human uterus was obtained by vaginal hysterectomy of patient A.B. at a local hospital. The patient, 27 years old, experienced menorhagia and desired permanent sterilization. The uterus had not been exposed to exogenous estrogens in the form of oral contraceptives and was in the seventh day of the menstrual cycle. Immediately after hysterectomy, the uterus was transported to the pathology laboratory for examination. The uterus was bisected in a sagittal plane. No pathological abnormalities were observed in the one-half of the uterus.

The other half of the uterus was placed in ice-cold buffer and transported to this laboratory.

The uterine tissue was cut into pieces approximately 2 x 2 x 2 mm in buffer at 4°. No attempt was made to separate the endometrium from the myometrium. Approximately 1.4 g lots of tissue were homogenized in 15-ml tubes at 4° in 3 volumes of buffer with a Polytron PT-10 (Brinkmann Instruments, Toronto, Ontario) at setting 4 using 3 homogenization periods of 20 sec, each followed by a 45 sec cooling period. The homogenates were pooled and centrifuged at 5,000 x g for 20 min at 4°. The supernatant was recentrifuged at 5,000 x g for 20 min at 4° and then at 100,000 x g for 90 min at 1°. The volume of the uterine cytosol was measured. An aliquot was removed for protein determination and competitive-binding protein assay. The remainder was stored in 5-ml aliquots in liquid nitrogen.

Protein was determined by the biuret method (26) using bovine serum albumin as the standard protein.

4. Competitive Protein Binding Assays

The binding activity of the fresh cytosols was assayed by adding approximately 16,000 cpm (mass 54 pg) of ^{3}H-E_2 in 200 µl of appropriate buffer to a series of 12 x 75 mm disposable glass tubes. After cooling the tubes to 4°, an aliquot of cytosol (50-500 µl) was added. The total volume in each tube (except that containing 500 µl of cytosol) was made up to 600 µl with appropriate buffer. The tubes were incubated either at 4° for 16 hrs or at 37° for 1 hr and then cooled to 4°. A 1.0-ml aliquot of chilled dextran-coated charcoal suspension

was added to each tube, which, after gentle vortexing was allowed to stand for 10 min at 4°. The tubes were centrifuged at 6,000 x g for 10 min. A 1.0-ml aliquot of the supernatant was transferred into a scintillation vial, dissolved in 10 ml of scintillator and counted to determine the amount of tritiated estrogen bound (cpm).

Competitive protein binding curves for each estrogen were determined in the following manner. A series of 9 tubes were set up. In order to simulate the average counts in the sample tubes prior to assay, an aliquot of the appropriate tritiated estrogen in absolute ethanol (^{3}H-E_1 and ^{3}H-E_2, 340 cpm in 16.6 µl; ^{3}H-E_3, 280 cpm in 14.9 µl) was added to each tube and evaporated to dryness. Tube 1 was the zero standard. Aliquots of appropriate buffer (20-100 µl) containing 10, 25, 50, 100, 250 and 400 pg of unlabelled estrogen were added to tubes 2-7, respectively. The total volume in tubes 1-7 was made up to 100 µl with appropriate buffer. A 200-µl aliquot of appropriate buffer containing the tritiated estrogen (^{3}H-E_1, 10,000 cpm (mass 54 pg); ^{3}H-E_3, 9,500 cpm (mass 61 pg) or 16,000 cpm (mass 102 pg)) was added to each tube. The tubes were vortexed and then cooled to 4°. An aliquot of cytosol (100 — 300 µl) was added to tubes 1-7. The total volume in tube 8, the assay blank, was made up with appropriate buffer to a volume equal to that contained in tubes 1-7. The total volume in tube 9, the total counts tube, was made up with appropriate buffer to a volume 1.0 ml greater than that contained in tubes 1-7. The tubes were incubated at 4° for 16 hrs. Those steps after incubation (dextran-coated charcoal treatment, centrifugation and preparation of a sample for counting) were performed on tubes 1-8 as described above. A 1.0-ml aliquot of tube 9

was added to a scintillation vial, dissolved in 10 ml of scintillator and counted.

Samples derived from the extraction and column chromatography of plasma estrogens were assayed as follows. A 100-µl aliquot of appropriate buffer and 200 µl of the appropriate tritiated estrogen-containing buffer solution were added to each tube. The tubes were vortexed and then cooled to 4°. After addition of the appropriate volume of cytosol, the tubes were incubated at 4° for 16 hrs. Those steps after incubation were performed as described earlier. The amount of estrogen in the radioassay sample was determined from the standard curve. This value was corrected for losses during the extraction and column chromatography procedures. The resulting value represented that amount of unlabelled estrogen present in the original 2-ml plasma sample.

CHAPTER III

RESULTS AND DISCUSSION

A. EXTRACTION RECOVERY

The addition of 1,000 cpm of each tritiated estrogen to be monitored to the plasma samples enabled the percent recovery of the extraction procedure to be calculated. The percent recoveries of each individual estrogen or all three estrogens from plasma samples containing various amounts of unlabelled estrogen(s) (0-1,000 pg) are shown in Table I. The mean recoveries from plasma for E_1, E_2, E_3 and E_{123} were 89% $\pm$ 3 (SD), 91% $\pm$ 2 (SD), 85% $\pm$ 3 (SD) and 86% $\pm$ 2 (SD), respectively and the mean overall recovery was 88% $\pm$ 4 (SD).

B. SEPARATION OF THE ESTROGENS BY COLUMN CHROMATOGRAPHY

The elution pattern of the estrogens from both the 800 and 900 mg Sephadex LH-20 columns were determined by applying the sample containing the tritiated estrogen; eluting with appropriate volumes of chromatographic solvent, counting the radioactivity in the eluates, and calculating the percentage of estrogen in each eluate based on the total amount of estrogen collected. The results for the 800 and 900 mg columns are shown in Figures 1 and 2, respectively. The percentage values indicated were the averages of several chromatographic runs performed on several different 800 and 900 mg columns.

While E_3 was well resolved from E_1 and E_2, the latter two estrogens were not completely resolved on either the 800 or 900 mg column. In order to obtain fractions that contained only E_1 or E_2, it

Table I

Extraction Recovery

Labelled Estrogen(s)	Amount of Unlabelled Estrogen(s) Added (pg	% Recovery	Labelled Estrogen(s)	Amount of Unlabelled Estrogen(s) Added (pg)	% Recovery
^3H-E_1	0	92	^3H-E_3	0	87
	100 E_1	91		100 E_3	84
	200 E_1	91		500 E_3	85
	300 E_1	88		1,000 E_3	85
	100 E_{123}	84		100 E_{123}	80
	200 E_{123}	88		200 E_{123}	82
	200 E_{123}	88		300 E_{123}	87
^3H-E_2	0	93	^3H-E_{123}	0	84
	100 E_2	90		100 E_{123}	87
	500 E_2	93		200 E_{123}	86
	1,000 E_2	94		300 E_{123}	85
	100 E_{123}	88			
	200 E_{123}	91			
	300 E_{123}	91			

FIGURE 1

ELUTION OF E_1, E_2 AND E_3 FROM AN 800 MG SEPHADEX LH-20 COLUMN

Legend

For each box: the left vertical line indicates the total volume of chromatographic solvent which had been added and allowed to pass through the column before collection of an eluate was begun; the right vertical line indicates the total volume of chromatographic solvent which had been added and allowed to pass through the column when the collection of this eluate was stopped; and the top horizontal line represents the percentage of indicated estrogen contained in this eluate.

FIGURE 1

ELUTION OF E_1, E_2 and E_3 FROM AN 800 MG SEPHADEX LH-20 COLUMN

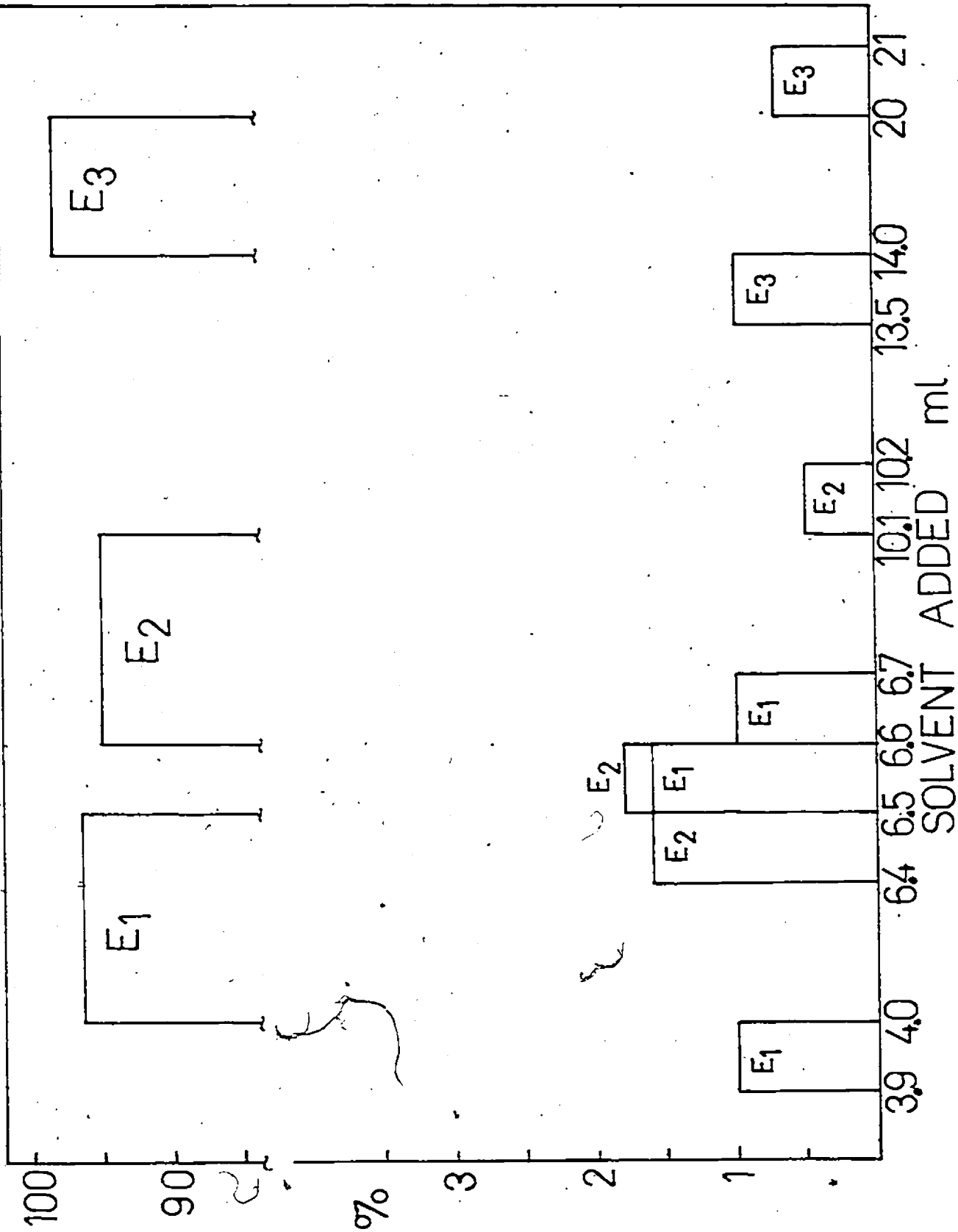


FIGURE 2

ELUTION OF E_1, E_2 AND E_3 FROM A 900 MG SEPHADEX LH-20 COLUMN

Legend

For each box: the left vertical line indicates the total volume of chromatographic solvent which had been added and allowed to pass through the column before collection of an eluate was begun; the right vertical line indicates the total volume of chromatographic solvent which had been added and allowed to pass through the column when the collection of this eluate was stopped; and the top horizontal line represents the percentage of indicated estrogen contained in this eluate.

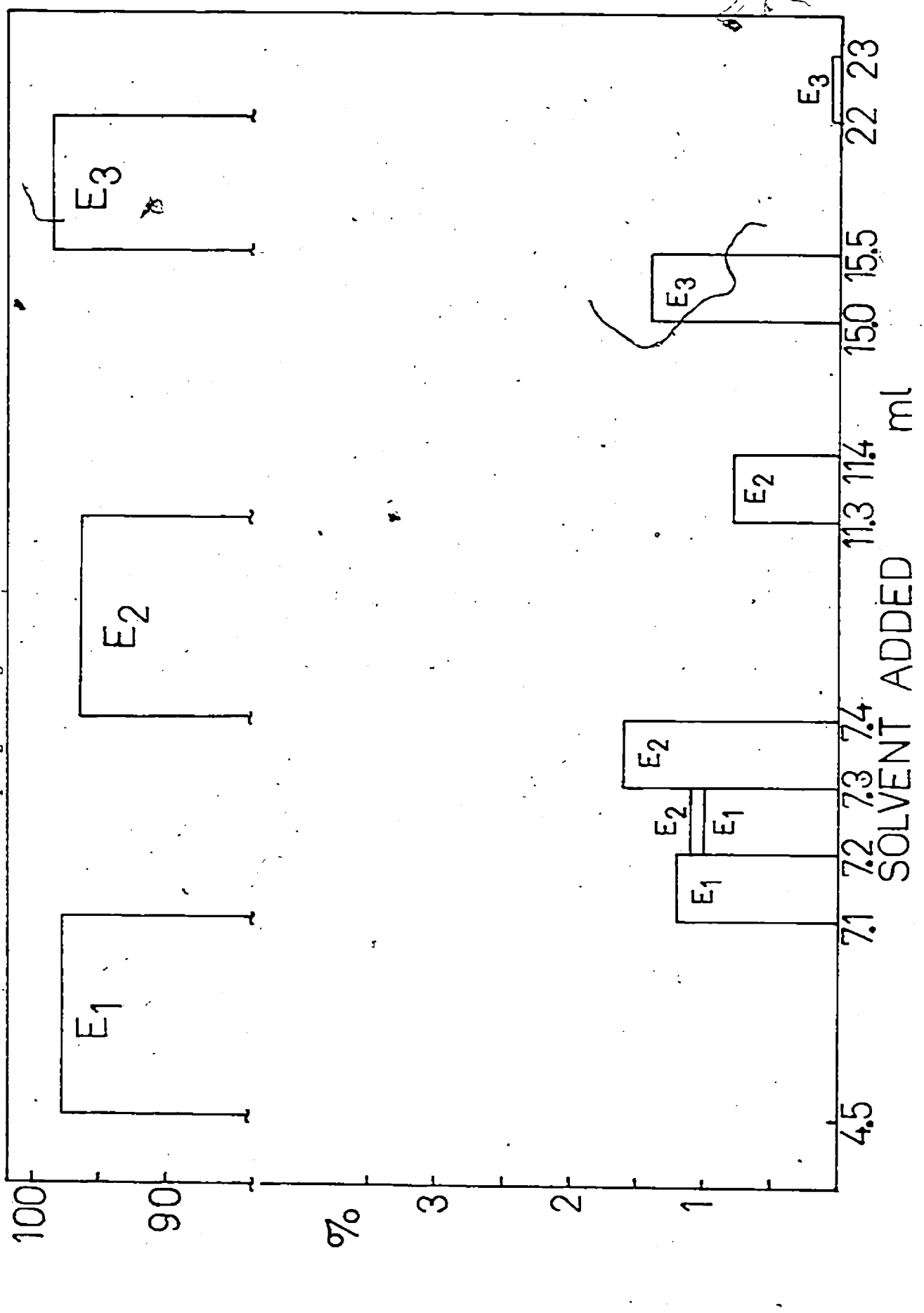


FIGURE 2

ELUTION OF E_1, E_2 AND E_3 FROM A 900 MG SEPHADEX LH-20 COLUMN

was necessary to stop E_1 collection early and to begin E_2 collection late. The elution scheme adopted for the 900 mg column was as follows: 4.6 ml, eluate discarded; 2.5 ml, eluate collected (E_1); 0.3 ml, eluate discarded; 4.0 ml, eluate collected (E_2); 3.6 ml, eluate discarded; 7.0 ml, eluate collected (E_3). Only 2-3% of the E_1 and E_2 are lost in the discarded 0.3-ml eluate. Significantly greater amounts of E_1 and E_2 would be lost if a similar system was adopted for use with the 800 mg column. One disadvantage of the 900 mg column was a reduced flow rate (approximately 0.4 ml/min compared with 0.5 ml/min) and larger elution volumes, both of which increased the time necessary for the chromatography.

No significant changes in the elution volume of any estrogen were observed in samples containing up to 1,000 pg of that estrogen or up to 300 pg of each estrogen.

C. EXTRACTION AND COLUMN CHROMATOGRAPHY RECOVERY

A series of 14 tubes were set up. A 2.0-ml aliquot of plasma and 50 µl of absolute ethanol containing 1,000 cpm of each tritiated estrogen were added to each tube. Tubes 1 and 2 were the sample blanks. i.e. containing only endogeneous estrogens. Aliquots of appropriate buffer containing 20, 50, 100, 200, 400 and 600 pg of each unlabelled estrogen were added to tubes 3 and 4, 5 and 6, 7 and 8, et cetera, respectively. Extraction and column chromatography on 900 mg columns were performed. The column eluates corresponding to the odd-numbered tubes were prepared for recovery determination and assay according to Method A,

while those of the even-numbered tubes were prepared according to Method 2 (Chapter II C 2). The percent recoveries are shown in Table II.

There was no statistical difference in the mean recovery or standard deviation between Methods A or B for any of the three estrogens. The overall mean recoveries for E_1, E_2, and E_3 were 85.3% ± 3.8, 84.3% ± 4.1 and 72.1 % ± 4.8, respectively.

D. COMPETITIVE PROTEIN BINDING ASSAYS

For each of the following experiments, information is given concerning the components of the incubation mixture (the mass of unlabelled estrogen, the radioactivity and mass of the tritiated estrogen, the volume and source of the cytosol) and the incubation temperature and duration. Complete procedural details have been described elsewhere (Chapter II C 4).

1. Calf Uterine Cytosols

Calf uterus A (52 g) afforded 99 ml of cytosol with a protein concentration of 7.3 mg/ml. Calf uterus B (47 g) afforded 145 ml of cytosol with a protein concentration of 8.3 mg/ml.

The binding activity of the fresh cytosols was initially assayed by incubating 16,000 cpm (mass 54 pg) of ^{3}H-E_2 with 50, 100 and 200 µl aliquots of both cytosols at 47° for 1 hr. The percent ^{3}H-E_2 bound for the 50, 100 and 200 µl samples of cytosol A were 1.6, 3.1 and 6.1%, respectively, and those for cytosol B were 1.8, 3,4 and 5.6%, respectively. There was a linear relationship between the amount of cytosol present in the incubation mixture and the amount of bound radio-

Table II

Extraction and Column Chromatography Recovery

Amount of Each Unlabelled Estrogen Added (pg)	% Recovery					
	E_1		E_2		E_3	
	Method A	Method B	Method A	Method B	Method A	Method B
0	78.0	87.7	77.1	80.9	65.3	76.3
20	89.8	88.0	84.8	85.6	78.1	67.5
50	88.3	83.6	84.8	81.8	79.1	70.0
100	85.1	81.8	86.3	85.1	73.8	74.5
200	91.7	82.6	95.2	83.0	75.6	75.9
400	83.7	85.1	84.8	80.4	72.3	64.9
600	87.9	80.9	87.3	83.7	66.5	70.1

activity. It was concluded that the low level of binding achieved during the 1-hr incubation would not provide sufficient sensitivity for the generation of accurate competitive protein binding curves.

Competitive protein binding curves for E_2 were generated using the fresh cytosols. Tubes containing E_2 (0-450 pg), 16,000 cpm (mass 54 pg) of ^{3}H-E_2 and 100 µl of cytosol were incubated at 4° for 16 hrs. The percent ^{3}H-E_2 bound ranged from 10.8% in zero standard to 3.8% in the 400 pg E_2 standard for cytosol A and 11.3% to 5.0%, respectively for cytosol B. On the basis of this limited sensitivity, it was concluded that an insufficient amount of receptor was contained in 100 µl of either cytosol.

Competitive protein binding curves for E_1, E_2 and E_3 were generated using thawed cytosol A. Tubes containing E_1, E_2 or E_3 (0-450 pg); 10,000 cpm (mass 53 pg) or ^{3}H-E_1, 16,000 cpm (mass 54 pg), or 9,500 cpm (mass 61 pg); and 300 µl of cytosol were incubated at 4° for 16 hrs. The total amount of tritiated estrogen bound (cpm) in the original incubation mixture volume was calculated for each standard tube:

$$\left[\begin{array}{l}\text{observed cpm}\\ \text{in 1.0 ml of the}\\ \text{standard tube}\end{array} - \begin{array}{l}\text{observed cpm}\\ \text{in 1.0 ml of the}\\ \text{assay blank}\end{array}\right] \times \begin{array}{l}\text{total dextran-coated}\\ \text{charcoal-incubation}\\ \text{mixture volume}\end{array}$$

The resulting data for each estrogen were handled in a variety of ways.

Plots of reciprocal of the amount of tritiated estrogen bound (cpm^{-1}) versus the amount of unlabelled estrogen (pg) added yielded approximately straight lines (Figure 3).

The data for each estrogen was normalized in the following

FIGURE 3

COMPETITIVE PROTEIN BINDING CURVE OF E_1, E_2, E_3

WITH CALF UTERINE CYTOSOL

Legend

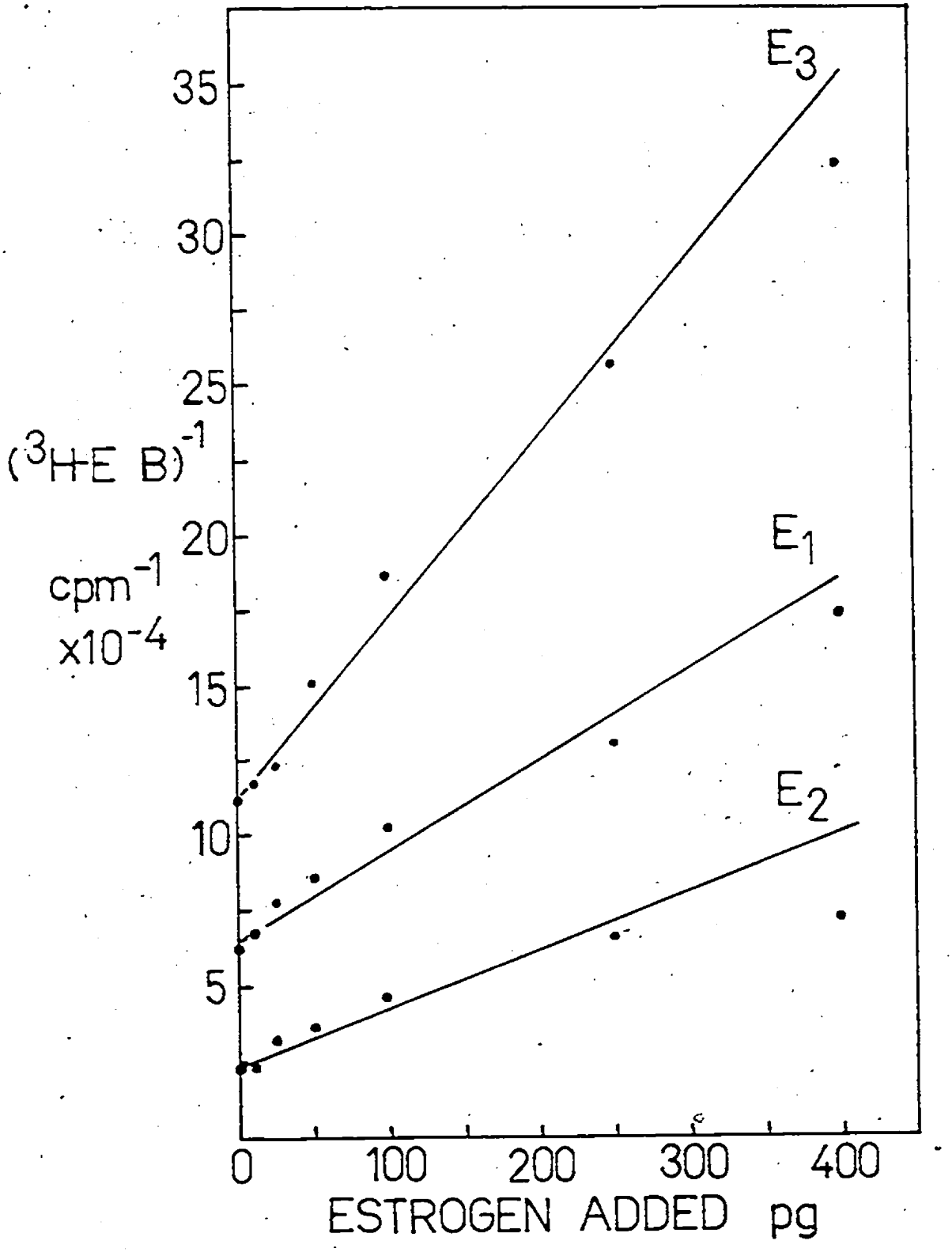
Tubes containing E_1, E_2 or E_3 (0-450 pg); 10,000 cpm (mass 53 pg) of ^{3}H-E_1, 16,000 cpm (mass 54 pg) of ^{3}H-E_2 or 9,500 cpm (mass 61 pg) of ^{3}H-E_3; and 300 µl of calf uterine cytosol were incubated at 4° for 16 hrs.

The reciprocal of the amount of tritiated estrogen bound $(^3\text{H-E B})^{-1}$ (cpm^{-1}) plotted against the amount of unlabelled estrogen added (pg) for E_1, E_2 and E_3.

FIGURE 5

COMPETITIVE PROTEIN BINDING CURVE OF E_1, E_2 E_3

WITH CALF UTERINE CYTOSOL



manner. The total amount of tritiated estrogen bound for each tube that contained added unlabelled estrogen was expressed as a percentage of that amount of tritiated estrogen bound in the zero standard. Plots of the percent tritiated estrogen bound normalized against the amount of unlabelled estrogen (pg) added and against the log of the amount of the unlabelled estrogen (pg) added are shown for each estrogen in Figures 4, 5, 6.

The actual percentage of tritiated estrogen bound at each level of unlabelled estrogen was calculated for each estrogen:

$$\frac{\text{total cpm of the standard tube} \times 100}{\left[\text{observed cpm in 1.0 ml of the total count tube} \times \text{total dextran-coated charcoal-incubation mixture volume}\right]}$$

The percent $^{3}H\text{-}E_2$ bound ranged from 23.1% in the zero standard to 8.8% in the 400 pg E_2 standard. The range for $^{3}H\text{-}E$ was 15.5% to 5.6% and for $^{3}H\text{-}E_3$, 8.4% to 3.1%.

A more rigorous analysis of the data for each estrogen was performed through the generation of Scatchard (27) plots. The following sequence of calculations were required on each standard tube for each estrogen.

First, the total amount of tritiated estrogen added in cpm was converted to dpm through knowledge of the counting efficiency and then converted to moles according to the specific activity of the tritiated estrogen:

$$\frac{\dfrac{\text{total estrogen cpm}}{\text{counting efficiency}} \times 100}{\text{specific activity (dpm/mole)}}$$

FIGURE 4

COMPETITIVE PROTEIN BINDING CURVE OF E_1

WITH CALF UTERINE CYTOSOL

Legend

Tubes containing E_1 (0-450 pg), 10,000 cpm (mass 53 pg) of ^3H-E_1 and 300 µl of calf uterine cytosol were incubated at 4° for 16 hrs. The percent ^3H-E_1 bound normalized (% ^3H-E_1 BN) is plotted against the amount of E_1 (pg) added ———— (———) and against the log of the amount of E_1 added (—— ——).

FIGURE 4

COMPETITIVE PROTEIN BINDING CURVE OF E_1

WITH CALF UTERINE CYTOSOL

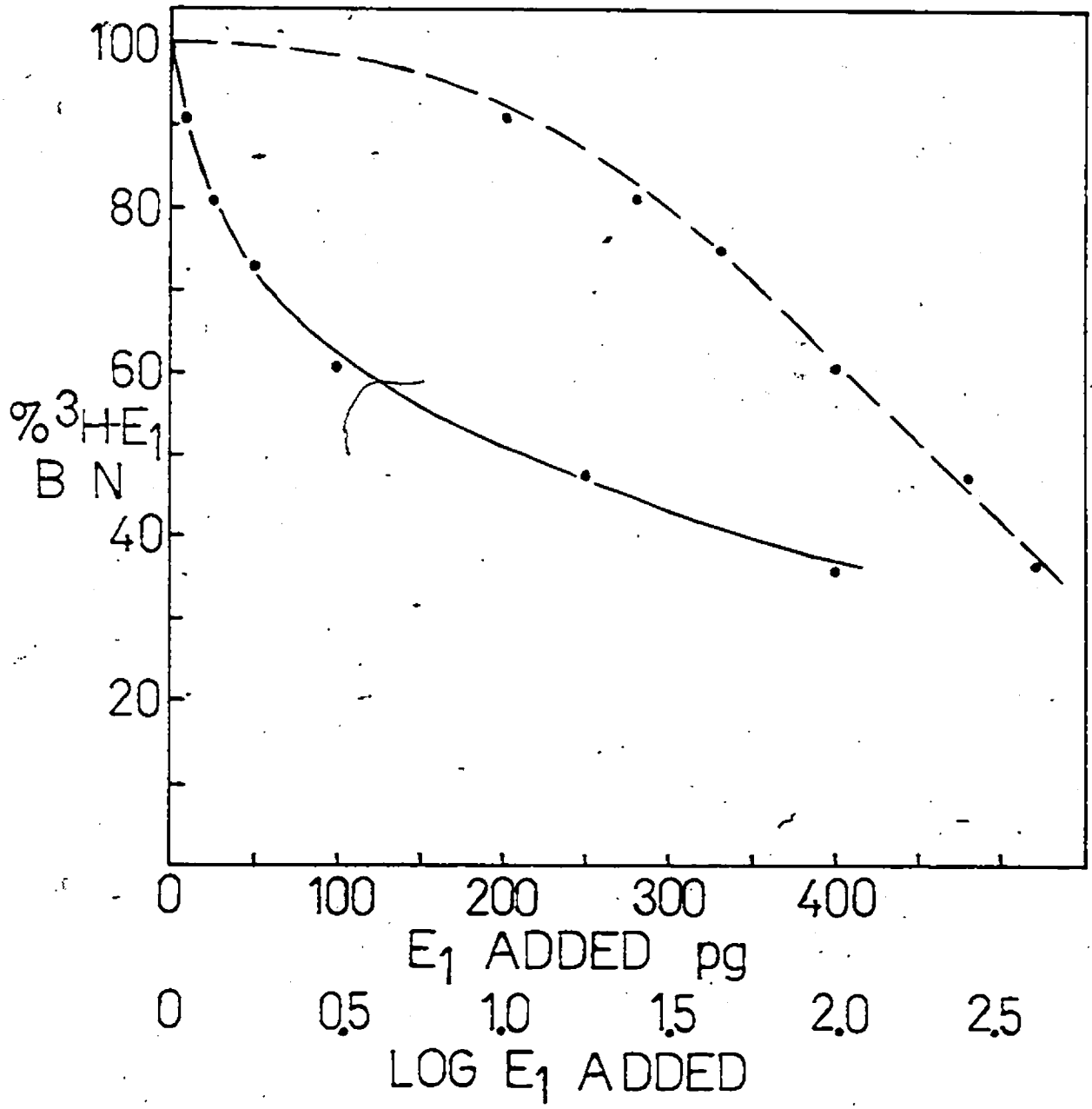


FIGURE 5

COMPETITIVE PROTEIN BINDING CURVE OF E_2

WITH CALF UTERINE CYTOSOL

Legend

Tubes containing E_2 (0-450 pg), 16,000 cpm (mass 54 pg) of ^3H-E_2 and 300 µl of calf uterine cytosol were incubated at 4°. for 16 hrs. The percent ^3H-E_2 bound normalized (% ^3H-E_2 BN) is plotted against the amount of the E_2 (pg) added ———— (— · —) and against the log of the amount of E_2 added (—— ——).

FIGURE 5

COMPETITIVE PROTEIN BINDING CURVE OF E_2 WITH CALF UTERINE CYTOSOL

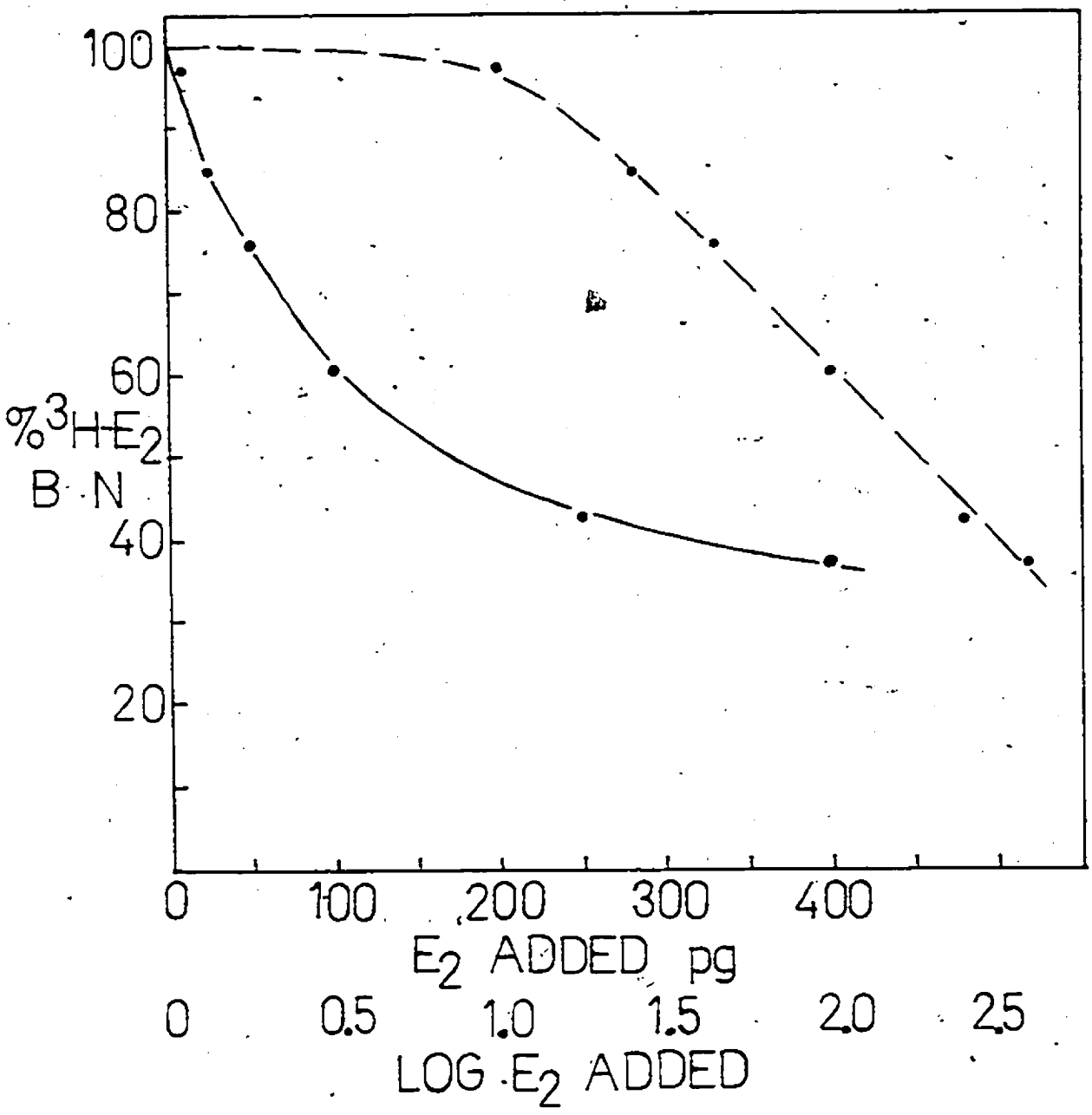


FIGURE 6

COMPETITIVE PROTEIN BINDING CURVE OF E_3

WITH CALF UTERINE CYTOSOL

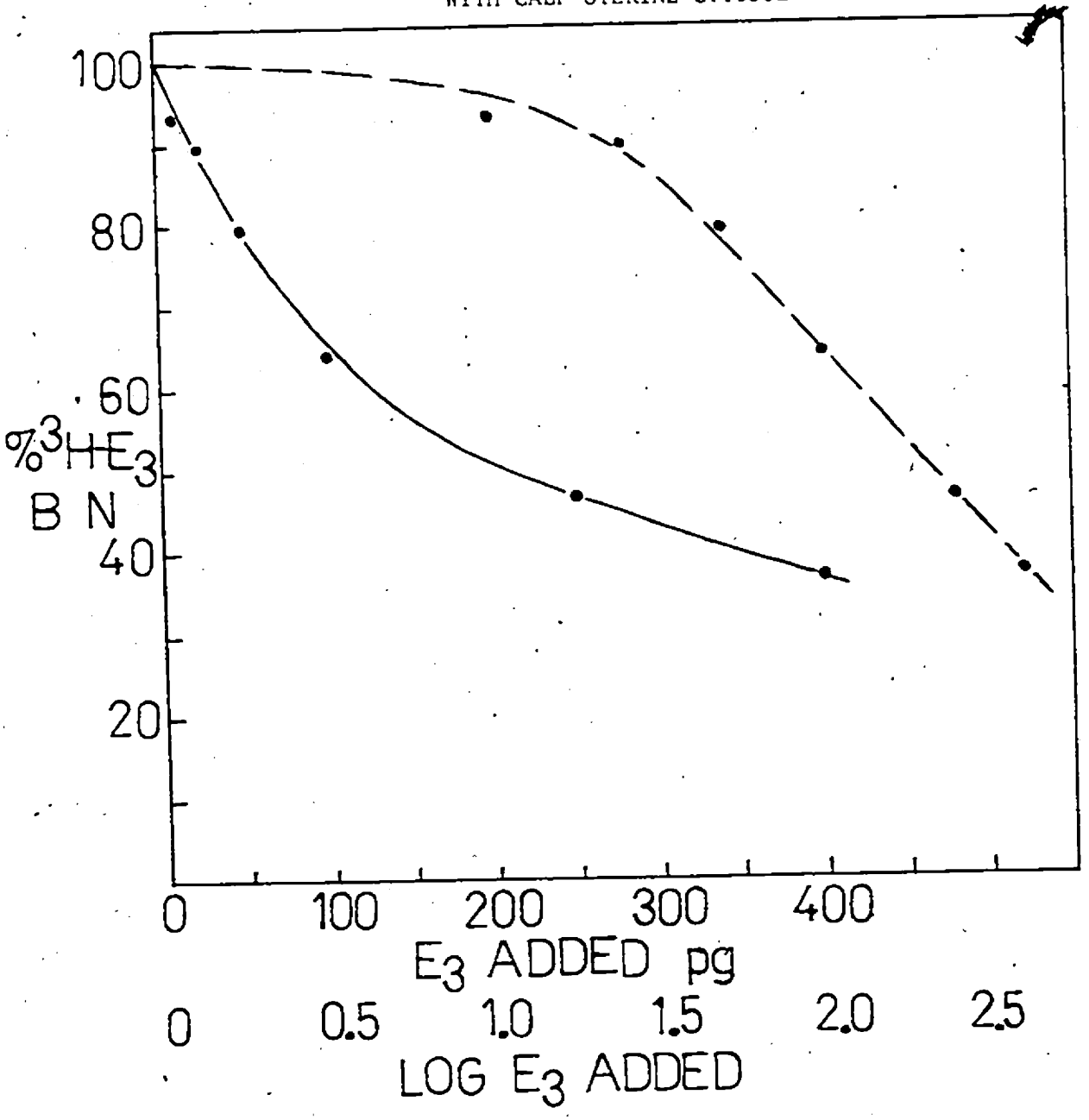
Legend

Tubes containing E_3 (9-450 pg), 9,500 cpm (mass 61 pg) of ^3H-E_3 and 300 µl of calf uterine cytosol were incubated at 4° for 16 hrs. The percent ^3H-E_3 bound normalized (% ^3H-E_3 BN) is plotted against the amount of the E_3 (pg) added ———— (———) and against the log of the amount of E_3 added (—— ——).

FIGURE 6

COMPETITIVE PROTEIN BINDING CURVE OF E_3

WITH CALF UTERINE CYTOSOL



The amount of unlabelled estrogen added was also converted to moles. Then the total number of moles of estrogen bound per x ml of incubation mixture (x = total volume of the incubation mixture) was calculated:

$$\begin{pmatrix}\%\text{ tritiated estrogen}\\ \text{bound per } x \text{ ml}\end{pmatrix} \times \left\{ \begin{pmatrix}\text{moles of tritiated}\\ \text{estrogen added}\end{pmatrix} + \begin{pmatrix}\text{moles of unlabelled}\\ \text{estrogen added}\end{pmatrix} \right\}$$

Since the protein concentration and the aliquot volume were known, the amount of protein (y mg) in the incubation mixture was calculated. Thus the answer to the previous calculation was re-expressed as the total number of moles of estrogen bound per y mg of protein per x ml of incubation mixture. This value was normalized to 1 mg of protein and 1 litre to give B, the total number of moles of estrogen bound per mg of protein per litre:

$$\begin{pmatrix}\text{total number of moles}\\ \text{of estrogen bound per}\\ y \text{ mg of protein per } x \text{ ml}\end{pmatrix} \times \frac{1}{y} \times \frac{1000}{x}$$

The total number of moles of estrogen free per x ml of incubation mixture was calculated by subtracting the total number of moles of estrogen bound per y mg of protein per x ml of incubation mixture from the total number of moles of estrogen (tritiated and unlabelled) added. This value was normalized to 1 litre to give F, the total number of moles of estrogen free per litre. The ratio B/F was calculated and then plotted against B. The results for E_1, E_2 and E_3 are represented by the solid line in Figures 7, 8 and 9, respectively.

Ideally the Scatchard plot should be a straight line. However, due to the limited number of high-affinity receptors, at sufficiently

FIGURE 7

SCATCHARD PLOT FOR E_1

WITH CALF UTERINE CYTOSOL

Legend

Tubes containing E_1 (0-450 pg), 10,000 cpm (mass 53 pg) of ^3H-E_1 and 300 µl of calf uterine cytosol were incubated at 4° for 16 hrs. A Scatchard plot was constructed from the data (——) and corrected for non-specific binding (—— ——).

FIGURE 7

SCATCHARD PLOT FOR E_1 WITH CALF UTERINE CYTOSOL

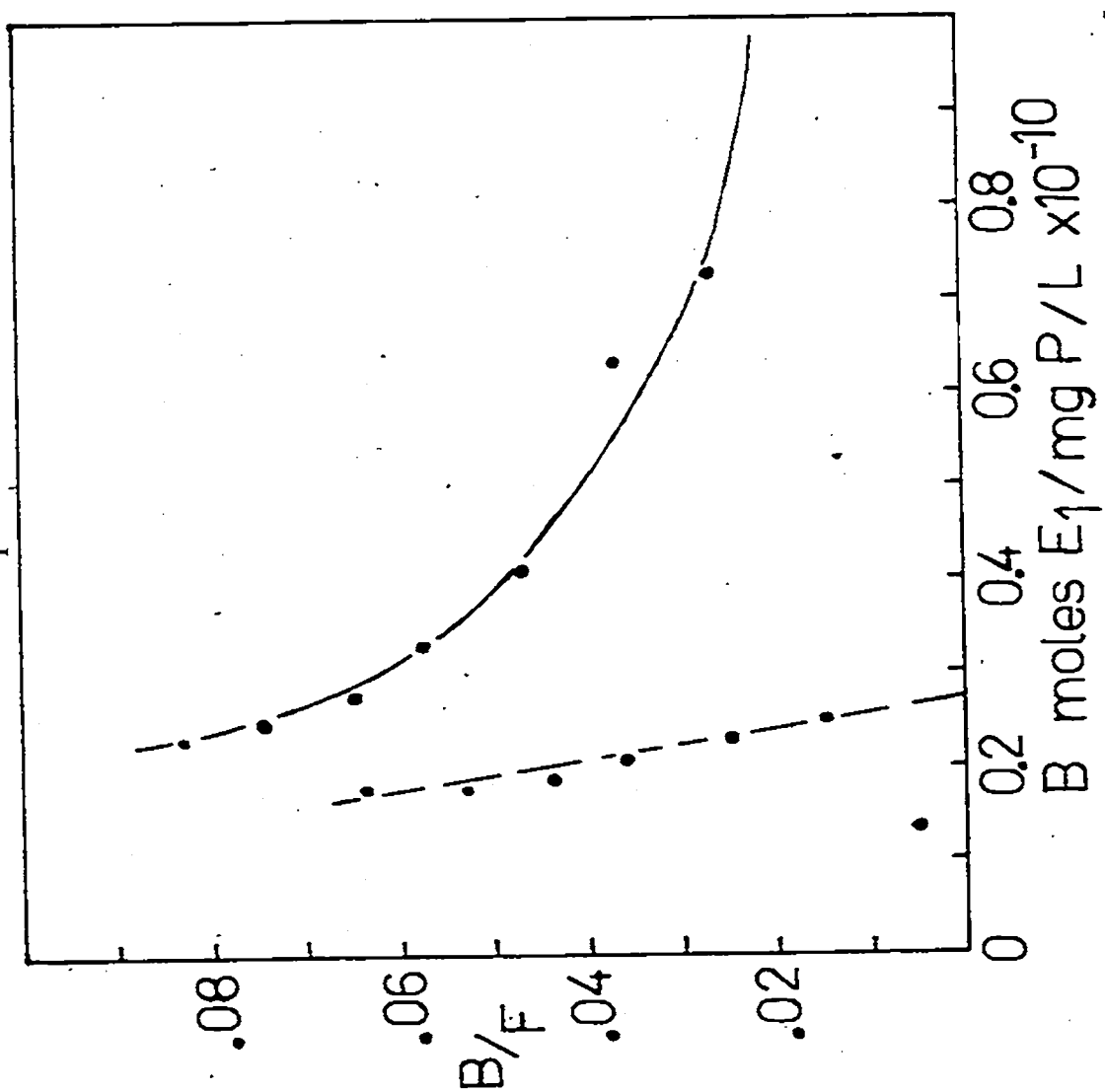


FIGURE 8

SCATCHARD PLOT FOR E_2

WITH CALF UTERINE CYTOSOL

Legend

Tubes containing E_2 (0-450 pg), 16,000 cpm (mass 54 pg) of ^3H-E_2 and 300 µl of calf uterine cytosol were incubated at 4° for 16 hrs. A Stachard plot was constructed from the data (———) and corrected for non-specific binding (—— ——).

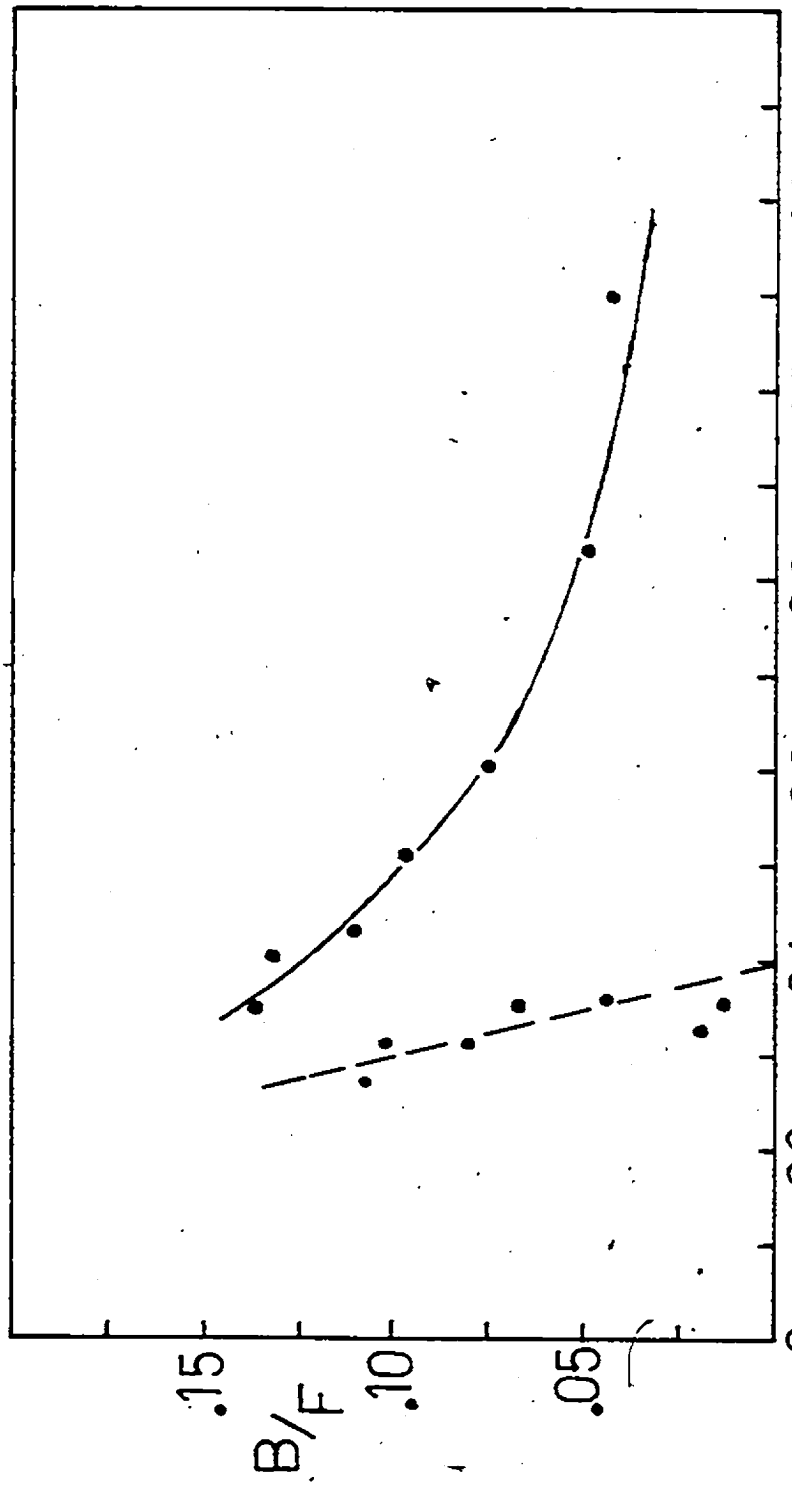


FIGURE 8

SCATCHARD PLOT FOR E_2 WITH CALF UTERINE CYTOSOL

FIGURE 9

SCATCHARD PLOT FOR E_3

WITH CALF UTERINE CYTOSOL

Legend

Tubes containing E_3 (0-450 pg), 9,500 cpm (mass 61 pg) of ^3H-E_3 and 300 µl of calf uterine cytosol were incubated at 4° for 16 hrs. A Scatchard plot was constructed from the data (———) and corrected for non-specific binding (—— ——).

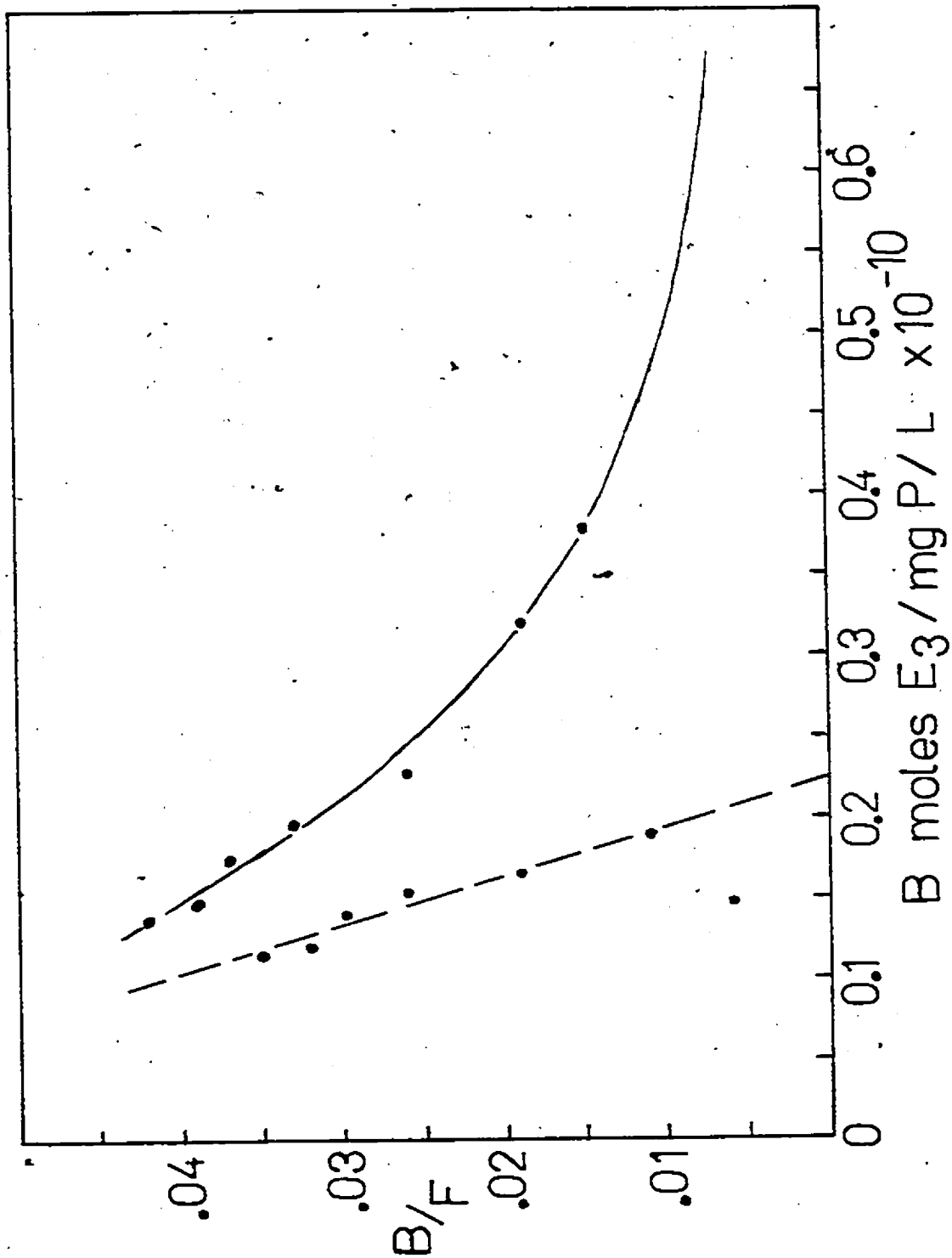


FIGURE 9

SCATCHARD PLOT FOR E_3 WITH CALF UTERINE CYTOSOL

high levels of estrogen a significant amount becomes bound to lower-affinity proteins present in the cytosol, such as albumin. This results in the tailing of the Scatchard plot at the lower end. This phenomenon was observed (Figures 5, 6, 7). The curves were corrected for this effect by the procedure of Chamness and McGuire (28). A tangent was drawn to the horizontal portion of the curve. The intercept on the B/F axis represented what is termed the B/F limiting ratio. The total number of moles of estrogen bound per mg protein per litre which were specifically bound to the high-affinity receptor (B_{sp}) was calculated for each point on the curve:

B - (F x B/F limiting ratio)

Then the ratio B_{sp}/F was calculated and then plotted against B_{sp} to produce the corrected line. This corrected line for E_1, E_2 and E_3 is represented by the dashed line in Figures 7, 8 and 9.

For the Scatchard plot, the slope of the line is equal to $-K_a$ (the association constant for the binding reaction and the B intercept is equal to the total molarity of the binding sites. The intercept value when multiplied by $x/1000$, when x equals the volume of the incubation mixture, is converted to the number of moles of receptor per mg protein and is referred to as M. The values of K_a and M for E_1, E_2 and E_3 were $6.0 \times 10^9\ M^{-1}$ and 1.7×10^{-14} moles/mg of protein, $1.4 \times 10^{10}\ M^{-1}$ and 2.4×10^{-14} moles/mg of protein, and $3.2 \times 10^9\ M^{-1}$ and 1.4×10^{-14} moles/mg of protein, respectively. As expected, the values of M were in relatively good agreement. However, the average value of M, 1.8×10^{-14} moles/mg of protein was approximately 30 times lower than

the maximum M of 3 - 6.5 x 10^{-13} moles/mg protein reported by Puca and Bresciani (29). The K_a for E_2 was in excellent agreement with the value of 1 x 10^{10} M^{-1} reported by Erdos (30).

It was concluded that the calf uterine cytosols contained insufficient receptor protein to be useful for competitive protein binding assay of unknown samples. Two factors are thought to have contributed to the low yield. The first was that the uteri had reduced receptor protein levels because they were too mature. Jungblut et al (31) recommended that uteri be obtained from 12-week old calves and that any uterus weighing more than 30 g be discarded. Neither of these conditions were fulfilled. Inadequate homogenization was believed to be the major factor.

Little or no specific binding activity remained in cytosol A which had been stored for 12 days at -20°C.

The extreme difficulty in obtaining immature calf liver uteri, and not the initial limited success, resulted in the change to human tissue.

2. Human Uterine Cytosol

The uterine tissue (approximately 27 g) afforded 80 ml of cytosol with a protein concentration of 6.4 mg/ml.

The binding activity of the fresh cytosols was initially assayed by incubating 16,000 cpm (mass 54 pg) of ^{3}H-E_2 with 50, 100, 200 and 500 µl of cytosol at 4° for 16 hrs. The percent ^{3}H-E_2 bound for the 50, 100, 200 and 500 µl samples were 13.2, 30.1, 54.7 and 77.3%, respectively.

Competitive protein binding curves for E_1, E_2 and E_3 were generated using cytosol which had been at 4° for 24 hrs. At this point, the remainder of the cytosol was placed in liquid nitrogen. Tubes containing E_1, E_2 or E_3 (0-450 pg); 14,000 cpm (mass 71 pg) of ^3H-E_1, 16,000 cpm (mass 54 pg) of ^3H-E_2, or 16,000 cpm (mass 102 pg) of E_3; and 200 µl of cytosol were incubated at 4° for 16 hrs.

All mathematical manipulations of the data were performed as described earlier. Plots of the reciprocal of the amount of tritiated estrogen bound (cpm^{-1}) versus the amount of estrogen (pg) added yielded straight lines (Figure 10). Plots of percent tritiated estrogen bound normalized versus the amount of unlabelled estrogen (pg) added and against the log of the amount of unlabelled estrogen (pg) added are shown for each estrogen in Figures 11, 12 and 13.

The percent ^3H-E_2 bound ranged from 40.4% in the zero standard to 10.6% in the 400 pg E_2 standard. The range for ^3H-E_1 was 25.2% to 9.2%, and for ^3H-E_3, 18.3% to 6.7%.

Scatchard plots for E_1, E_2, E_3 (Figures 14, 15, 16) were made and corrected in the case of E_1 and E_2. The values of K_a and M for E_1 E_2 and E_3 were 2.5×10^9 M^{-1} and 8.1×10^{-14} moles/mg of protein; 7.0×10^9 M^{-1} and 9.5×10^{-14} moles/mg of protein; 1.5×10^9 M^{-1} and 11.0×10^{-14} moles/mg of protein, respectively. The value of K_a for E_2 and the average value of M (1.0×10^{-13} moles/mg of protein) compared favourably with a K_a of 1×10^{10} M^{-1} and M of 2.85×10^{-13} moles/mg of protein reported by Henderson and Schalch (32). Their values were also determined using cytosol prepared from a non-pregnant uterus which had

FIGURE 10

COMPETITIVE PROTEIN BINDING CURVE OF E_1, E_2 AND E_3 WITH HUMAN UTERINE CYTOSOL

Legend

Tubes containing E_1, E_2 and E_3; 14,000 cpm (mass 71 pg) of ^{3}H-E_1, 16,000 cpm (mass 54 pg) or 16,000 cpm (mass 102 pg) of ^{3}H-E_3; and 200 µl of human uterine cytosol were incubated at 4° for 16 hrs. The reciprocal of the amount of tritiated estrogen bound $(^3\text{H-E B})^{-1}$ (cpm^{-1}) is plotted against the amount of unlabelled estrogen added (pg) for E_1, E_2 and E_3.

FIGURE 10

COMPETITIVE PROTEIN BINDING CURVE OF E_1, E_2 AND E_3 WITH HUMAN UTERINE CYTOSOL

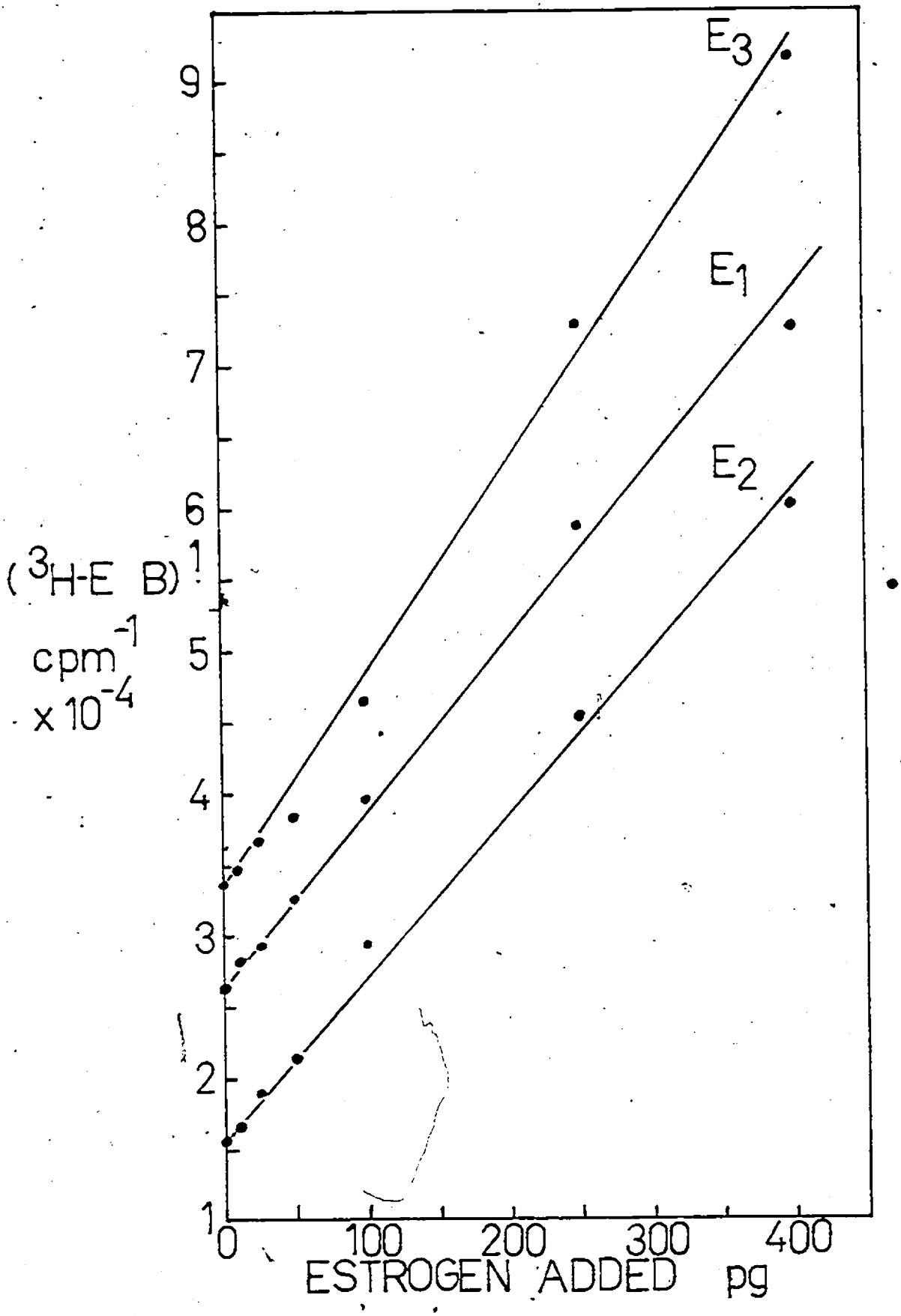


FIGURE 11

COMPETITIVE PROTEIN BINDING CURVE OF E_1

WITH HUMAN UTERINE CYTOSOL

Legend

Tubes containing E_1 (0-450 pg), 14,000 cpm (mass 71 pg) of ^3H-E_1 and 200 µl of human uterine cytosol were incubated at 4° for 16 hrs. The percent ^3H-E_1 bound normalized (% ^3H-E_1 BN) is plotted against the amount of E_1 (pg) added (———) and against the log of the amount of E_1 (pg) added (—— ——)

FIGURE 11

COMPETITIVE PROTEIN BINDING CURVE OF E_1

WITH HUMAN UTERINE CYTOSOL

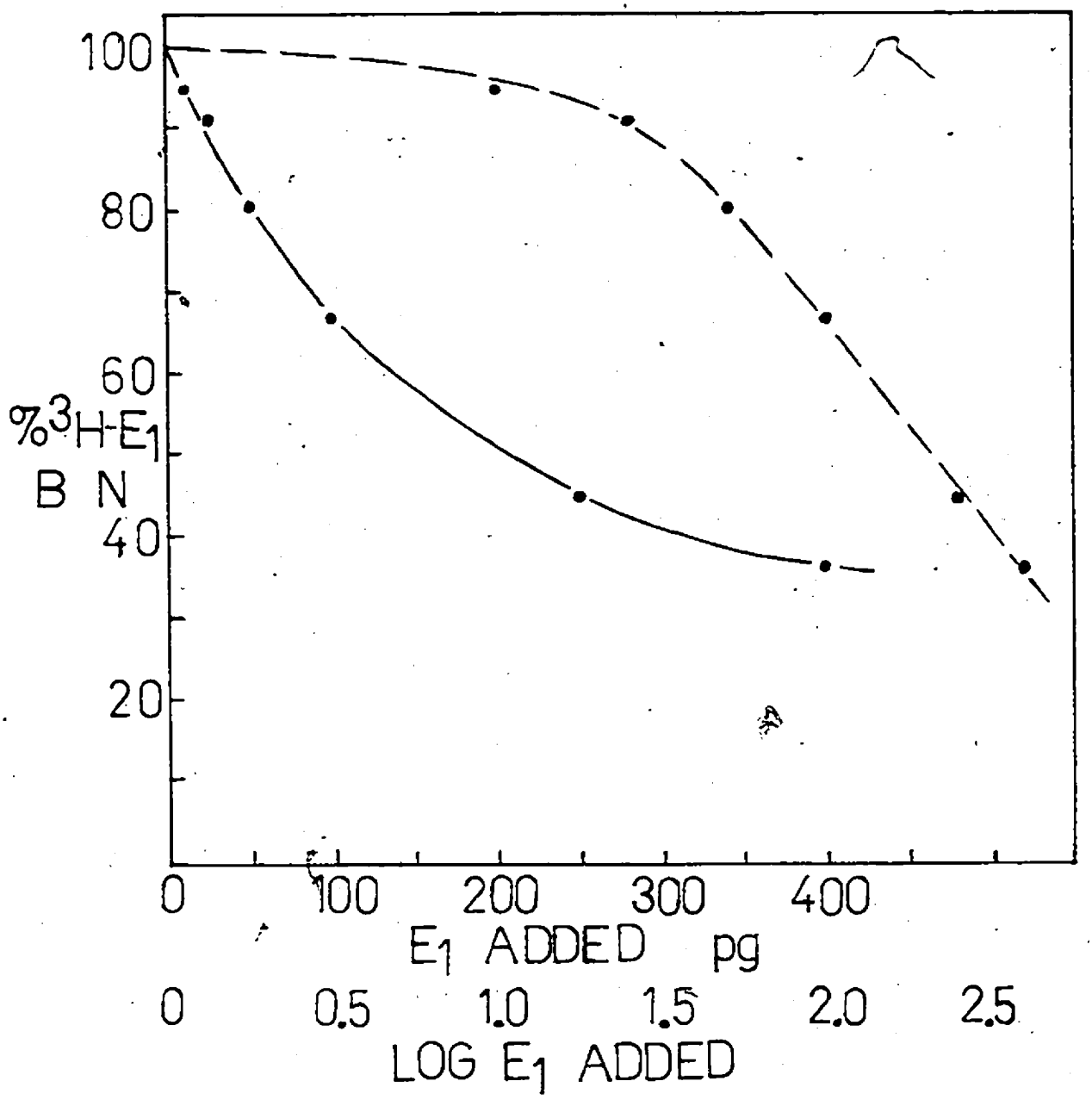


FIGURE 12

COMPETITIVE PROTEIN BINDING CURVE OF E_2

WITH HUMAN UTERINE CYTOSOL

Legend

Tubes containing E_2 (0-450 pg), 16,000 cpm (mass 54 pg) of ^3H-E_2 and 200 µl of human uterine cytosol were incubated at 4° for 16 hrs. The percent ^3H-E_2 bound normalized (% ^3H-E_2 BN) is plotted against the amount of E_2 added (———) and against the log of the amount of E_2 (pg) added (—— ——).

FIGURE 12

COMPETITIVE PROTEIN BINDING CURVE OF E_2

WITH HUMAN UTERINE CYTOSOL

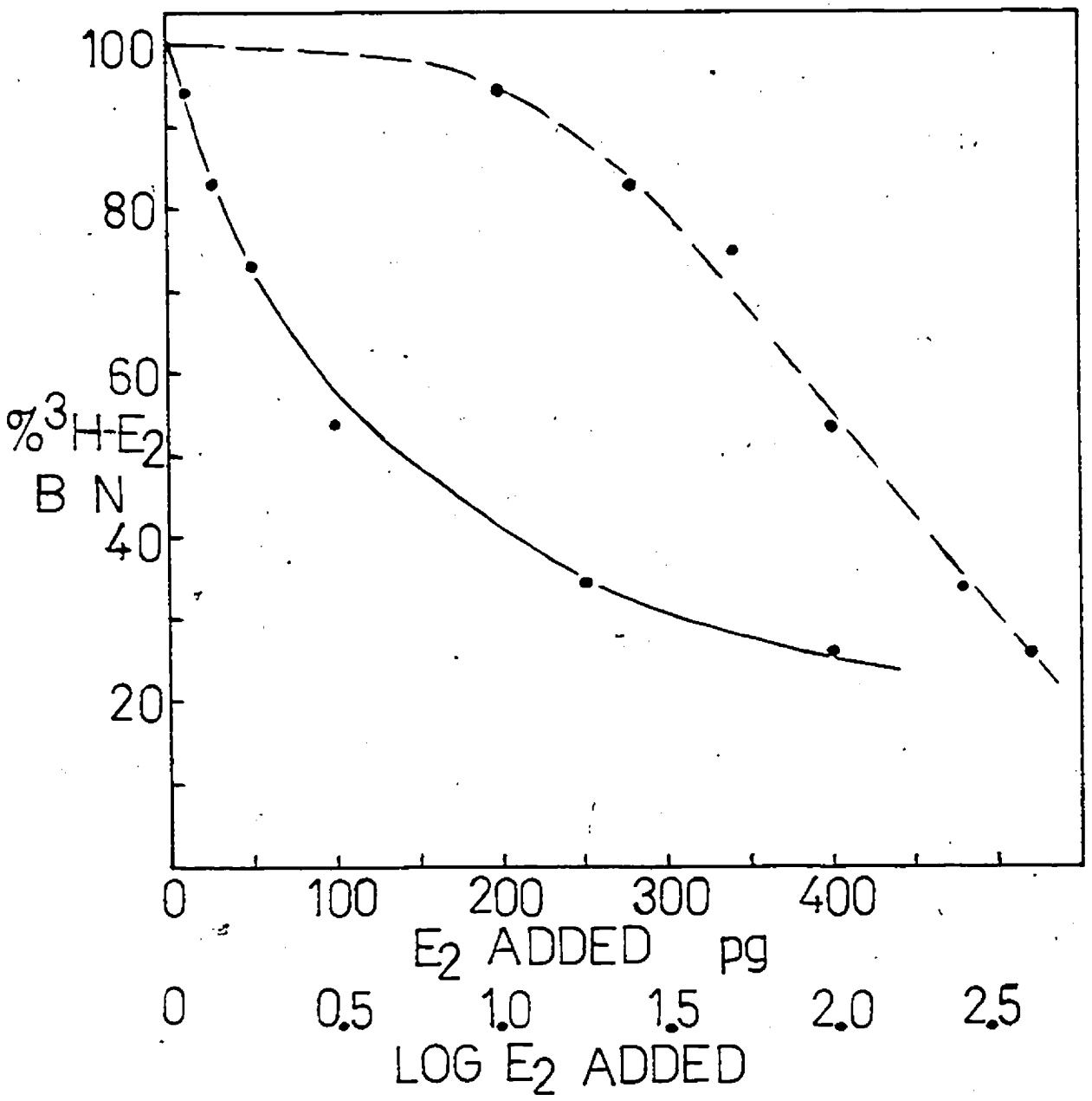


FIGURE 13

COMPETITIVE PROTEIN BINDING CURVE OF E_3

WITH HUMAN UTERINE CYTOSOL

Legend

Tubes containing E_3 (0-450 pg), 16,000 cpm (mass 102 pg) of ^3H-E_3 and 200 µl of human uterine cytosol were incubated at 4° for 16 hrs. The percent ^3H-E_3 bound normalized (% ^3H-E_3 BN) is plotted against the amount of E_3 added (———) and against the log of the amount of E_3 added (—— ——).

FIGURE 13

COMPETITIVE PROTEIN BINDING CURVE OF E_3

WITH HUMAN UTERINE CYTOSOL

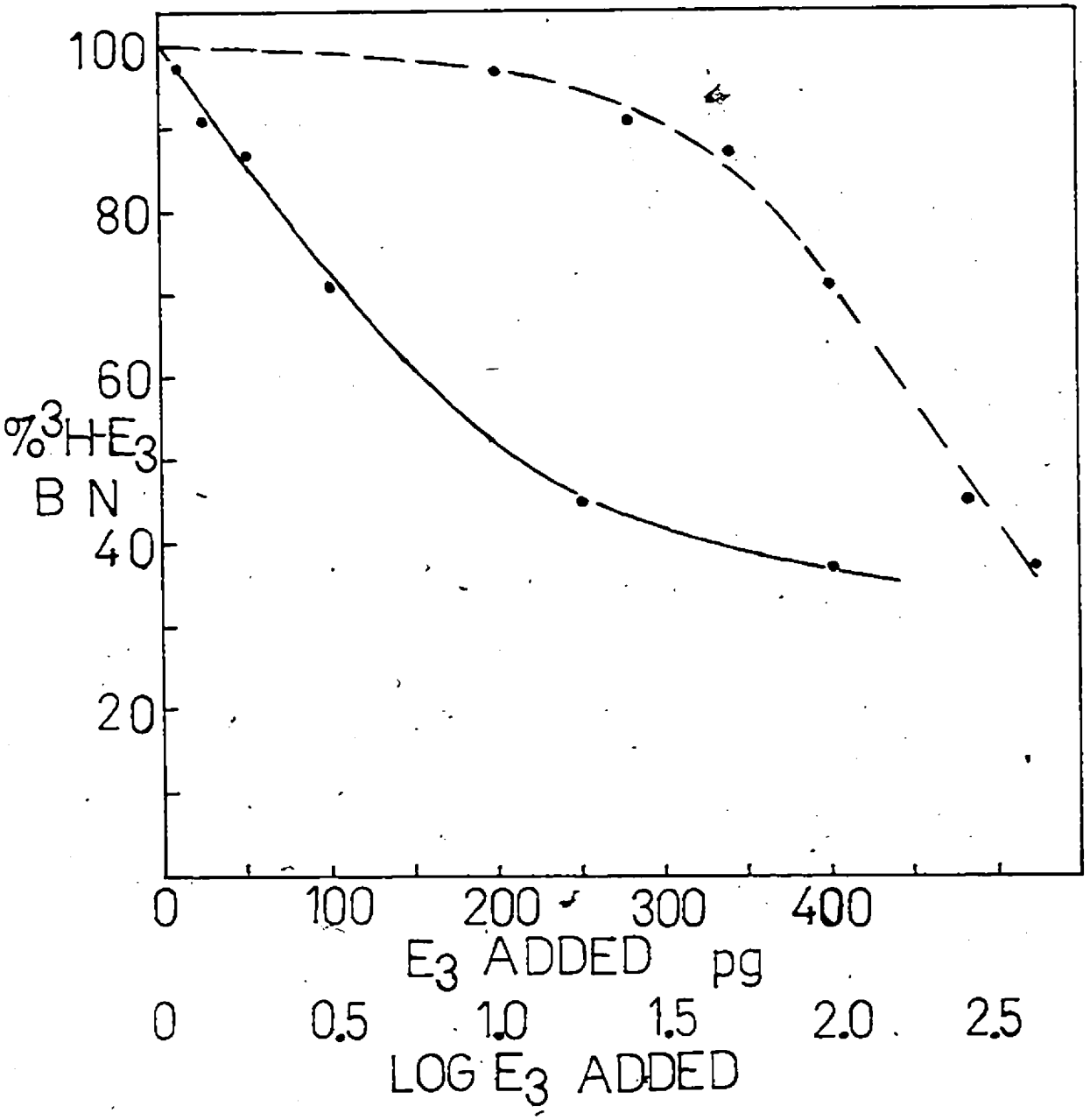


FIGURE 14

SCATCHARD PLOT FOR E_1

WITH HUMAN UTERINE CYTOSOL

Legend

Tubes containing E_1 (0-450 pg), 14,000 cpm (mass 71 pg) of ^3H-E_1 and 200 µl of human uterine cytosol were incubated at 4° for 16 hrs. A Scatchard plot was constructed from the data (———) and corrected for non-specific binding (—— ——).

FIGURE 14

SCATCHARD PLOT FOR E_1 WITH HUMAN UTERINE CYTOSOL

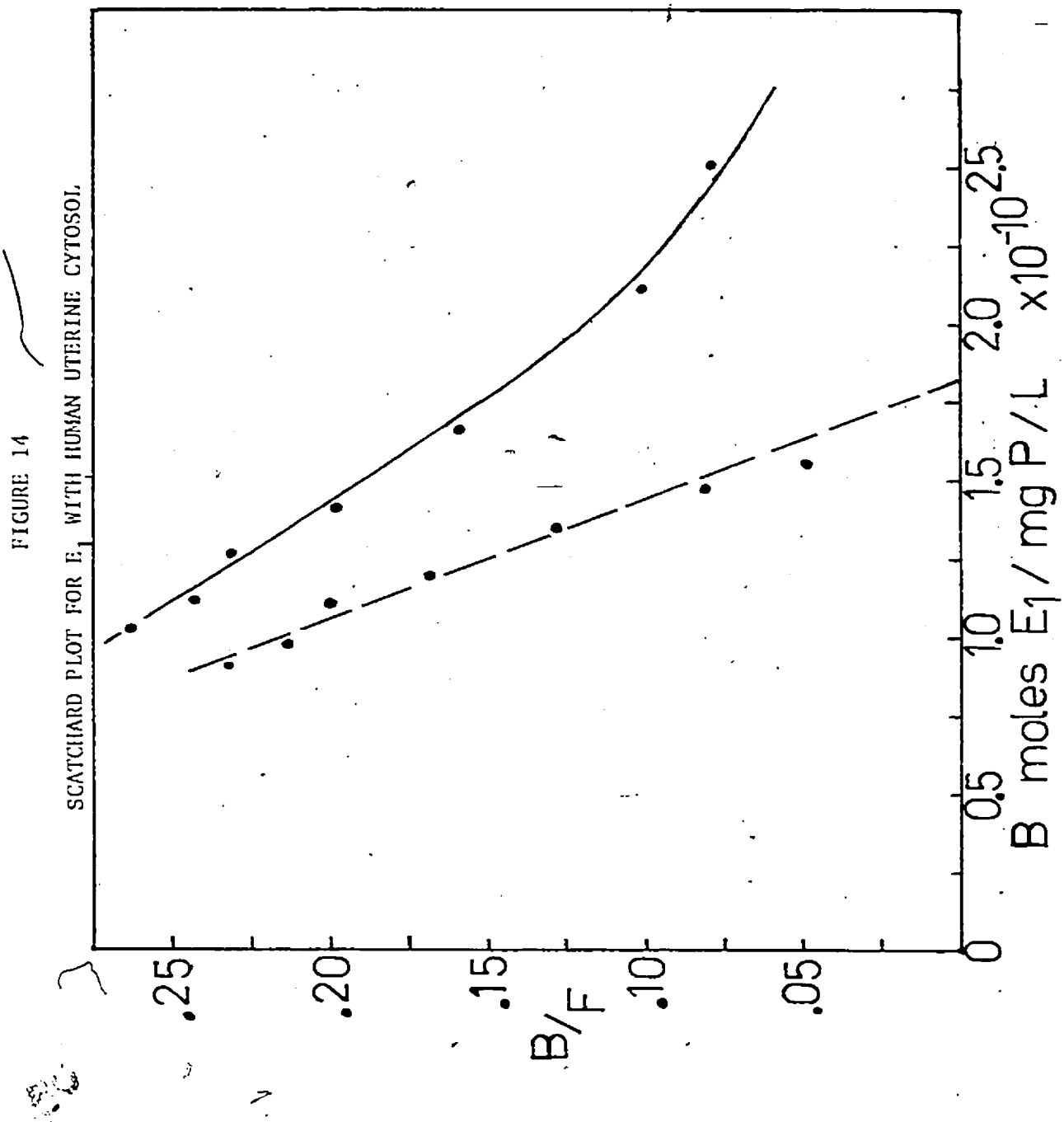


FIGURE 15

SCATCHARD PLOT FOR E_2

WITH HUMAN UTERINE CYTOSOL

Legend

Tubes containing E_2 (0-450 pg), 16,000 cpm (mass 54 pg) of ^3H-E_2 and 200 µl of human uterine cytosol were incubated at 4° for 16 hrs. A Scatchard plot was constructed from the data (———) and corrected for non-specific binding (—— ——).

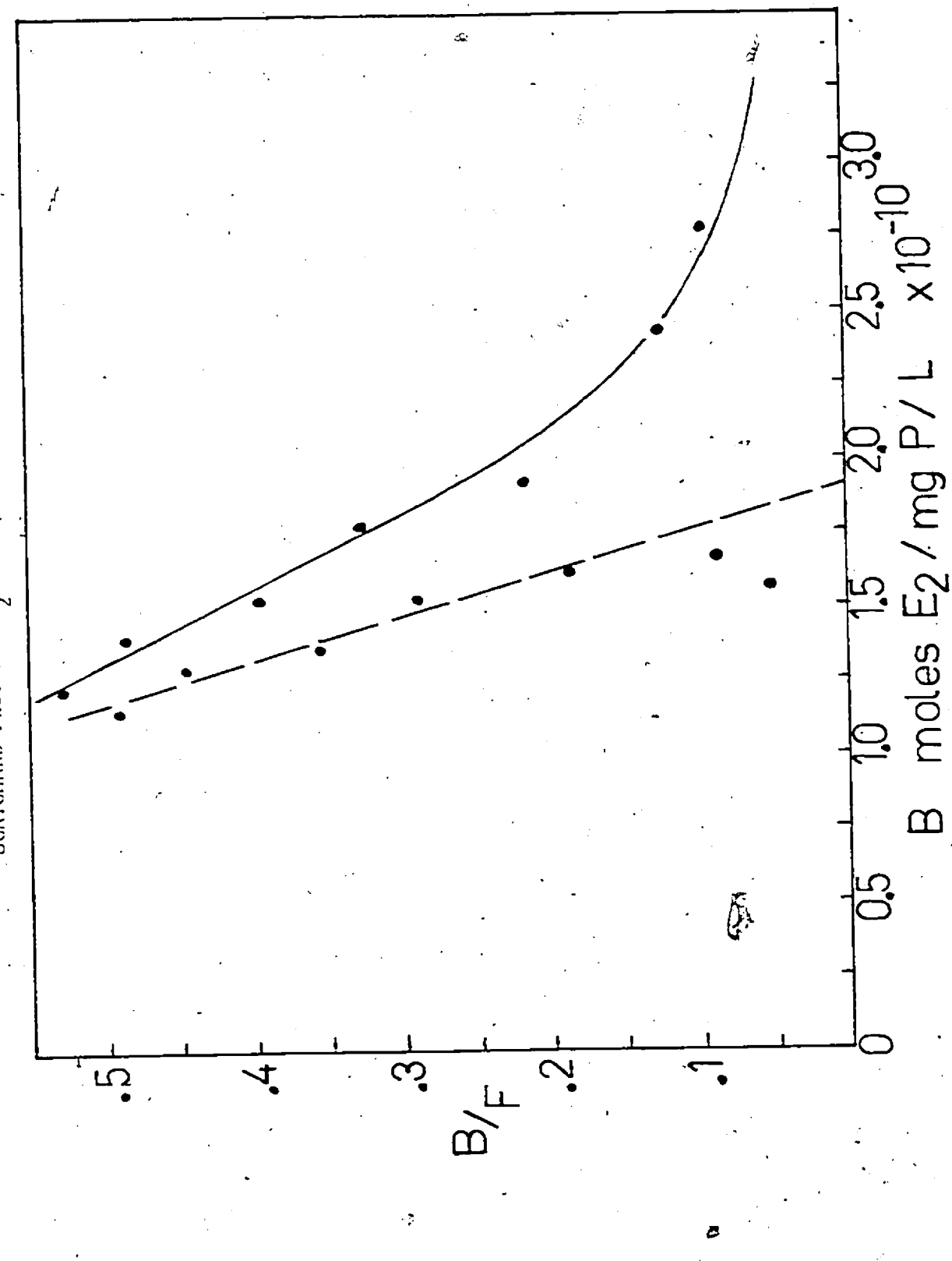


FIGURE 15

SCATCHARD PLOT FOR E_2 WITH HUMAN UTERINE CYTOSOL

FIGURE 16

SCATCHARD PLOT FOR E_3

WITH HUMAN UTERINE CYTOSOL

Legend

Tubes containing E_3 (0-450 pg), 16,000 cpm (mass 102 pg) of ^3H-E_3 and 200 µl of human uterine cytosol were incubated at 4° for 16 hrs. A Scatchard plot was constructed from the data (———).

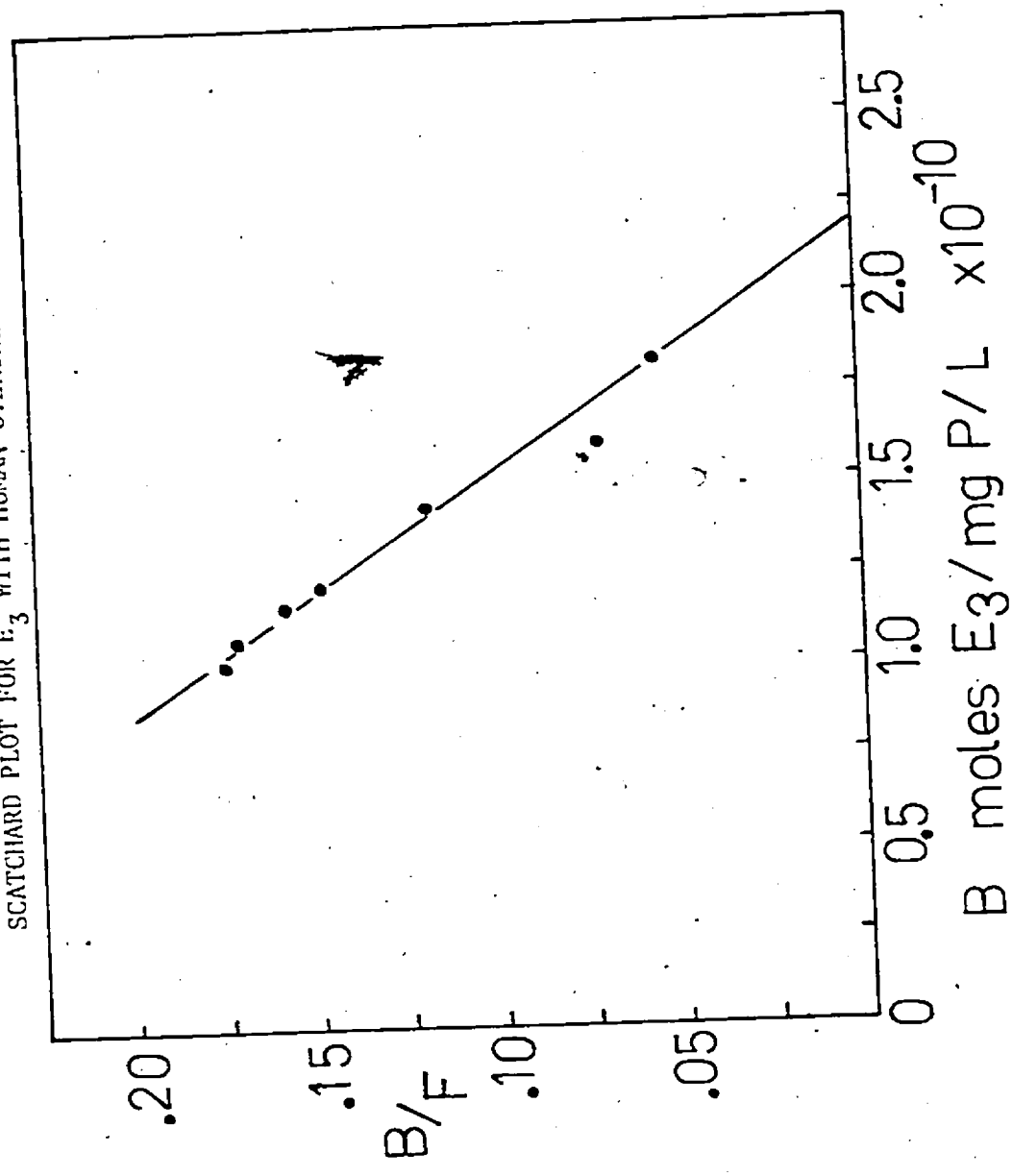


FIGURE 16

SCATCHARD PLOT FOR E_3 WITH HUMAN UTERINE CYTOSOL

not been exposed to exogeneous estrogens in the form of oral contraceptives.

The human uterine cytosol contained approximately four times as many moles of estrogen receptors/mg of protein than the calf uterine cytosol. The affinity of the human receptor for all three estrogens was slightly smaller than the calf receptor.

The human uterine cytosol appeared to have sufficient binding capacity to be used in the radioassay of samples derived from plasma. Any one of the graphs (cpm^{-1} vs E_2 added (pg); percent tritiated estrogen bound versus E_2 added (pg), or versus log E_2 added) appeared to be adequate for the standard curve.

The competitive protein binding curve for E_2 has been generated during two other experiments. In one case, the cytosol used had been stored for 7 days, and in the other case for 12 days. No decrease in binding activity whatsoever was observed. In fact, binding activity appeared to have increased slightly. Table III shows the percentages of ^3H-E_2 bound in each tube for the three experiments.

3. Assay of Samples Derived from the Extraction and Chromatography of Plasma Estrogens

Samples containing E_2 which had been prepared for assay during the extraction and column chromatography recovery experiment (Chapter III C) were incubated with 16,000 cpm (mass 54 pg) of ^3H-E_2 and 200 µl of human uterine cytosol at 4° for 16 hrs.

The results of the samples from the odd numbered tubes, the column eluates of which were prepared for recovery determination and

Table III

Stability of the Human Uterine Cytosol Stored in Liquid Nitrogen

Unlabelled Estrogen Added (pg)	% ^{3}H-E_2 Bound		
	Cytosol 0 days	Cytosol 7 days	Cytosol 12 days
0	40.4	44.5	47.7
10	38.1	41.6	44.2
25	33.5	36.7	41.2
50	29.5	32.8	34.1
100	21.7	24.6	24.0
250	13.9	15.6	14.4
400	10.6	11.8	11.2

assay by Method A, i.e., simply aliquoting approximately 40% of the eluate into each of two assay tubes and evaporating to dryness, are shown in Table IV. The experimental value for the amount of E_2 in the samples was determined by expressing each value of amount of ^3H-E_2 bound (cpm) as a percentage of the amount of ^3H-E_2 bound (cpm) in the zero standard and reading the value for the amount of E_2 (pg) off the percent ^3H-E_2 bound normalized versus the amount of E_2 (pg) added curve which had been prepared from the data of the standards. The calculated value for the amount of E_2 (pg) in the samples was calculated based on the known percent recovery. The amount of ^3H-E_2 bound in the assay tubes was generally a great deal higher than that observed in the standard tubes and consequently the experimentally determined values for the amount of E_2 (pg) in the assay tubes were low. These results were indicative of considerable non-specific binding by components which must have eluted from the column together with the E_2. The tubes containing no exogeneous E_2 were in reasonable agreement with the zero standard. This observation will be discussed later.

The results of the samples from the even-numbered tubes, the column eluates of which were prepared for recovery determination and assay by Method B, i.e., evaporating to dryness, redissolving in ethanol, adding to the two assay tubes equal volume aliquots (approximately 80% total) of the ethanol solution, and evaporating to dryness, are shown in Table V. The experimental and calculated values for the amount of E_2 (pg) in the samples were determined as described for the previous samples. Except the two assay tubes found to contain an average of 54

Table IV

Assay of E_2 Samples Prepared After Elution by Method A

Aliquot 1			Aliquot 2			Standards	
Calculated E_2 (pg)	^3H-E_2 Bound (cpm)	Experimental E_2 (pg)	Calculated E_2 (pg)	^3H-E_2 Bound (cpm)	Experimental E_2 (pg)	E_2 Added (pg)	^3H-E_2 Bound (cpm)
0	7040	0	0	7027	0	0	6899
6	8278	-	7	8453	-	10	6443
15	8038	-	18	7664	-	25	5689
30	6977	-	37	6314	12	50	5088
66	5718	36	81	5163	45	100	3815
118	4264	78	145	3535	118	250	2417
182	3440	123	223	2726	185	400	1829

Table V

Assay of Samples Prepared After Elution by Method B

Aliquots			Standards		
^3H-E_2 Bound (cpm)	^3H-E_2 Bound (cpm)	Average Experimental E_2 (pg)	Calculated E_2 (pg)	E_2 Added (pg)	^3H-E_2 Bound (cpm)
7572	7899	-	0	0	7387
7559	7753	-	7	10	6854
7436	7510	-	16	25	6381
6340	6859	12	34	50	5278
5420	4969	54	52	100	3715
3967	4572	78	127	250	2229
3641	3420	122	197	400	1741

pg (48 pg and 59 pg) of E_2, the experimental values were lower than calculated values due to non-specific binding. The two sets of assay tubes that resulted in values which were in reasonable agreement with the expected results had one characteristic in common. The ether extracts from which these assay tubes were derived were the only two of the 14 which had been chromatographed using freshly prepared Sephadex LH-20 columns. It was concluded that the ether extracts contained components capable of non-specific binding of estrogens and that these components were eluted from the column not during the initial, but rather during the subsequent chromatographic run. Routinely columns were washed between samples with approximately 10 ml of chromatographic solvent. It would be faster to prepare a fresh column than to wash with increased volumes of solvent. Perhaps another solvent could be found to readily remove these components. This problem was not detected prior to assay, since the presence of the components did not adversely affect the separation of the estrogens in subsequent samples.

No E_1 or E_3 assays have as yet been performed.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Evidence to the fact that a reliable method for the determination of plasma estrogens is close at hand comes from the observation that the experimental values for two sets of assay tubes were in excellent agreement with the expected values. Hopefully all of the potential hazards have been identified and only techniques which will improve results remain to be discovered.

The extraction and column chromatographic procedures have been well documented. It is imperative that a freshly prepared or "clean" Sephadex LH-20 column be used for each sample. Optimal binding activity is achieved through efficient tissue homogenization and is best maintained by storage of the cytosol in liquid nitrogen.

Future studies include: confirmation of the hope that the method can be used to accurately and precisely determine plasma estrogens; establishment of the lower limits of detection; and extension of the principles of this assay to determine conjugated plasma estrogens and to determine the number of estrogen receptors in tissues.

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